University of Alberta

IDENTIFICATION OF NOVEL COMPONENTS OF THE DYSTROPHIN GLYCOPROTEIN COMPLEX

BY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry

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ABSTRACT

Dystrophin is the protein product of the Duchenne Muscular Dystrophy gene. The dystrophin protein consists of an N-terminus, a rod domain, a cysteine rich region and a C-terminus. The yeast two-hybrid assay was performed to identify proteins interacting with the C-terminus of dystrophin. Using this system α-actinin-2 and α-actin were identified as new dystrophin binding proteins. These interactions were confirmed by ligand blotting technique, co-localization and immunolocalization of dystrophin and α-actinin in muscle cells. One of the proteins in the Dystrophin Glycoprotein Complex, α-sarcoglycan, was also examined for protein-protein interactions using the yeast two hybrid system and was shown to interact with dbpA and a fragment of the Na^+/K^+ ATPase. These newly discovered protein-protein interactions may form a communication link between the cytoskeleton and the extracellular matrix.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ab	antibody
AD	activation domain
Ag	antigen
amp	ampicillin
ATP	adenosine triphosphate
BD	binding domain
BMD	becker muscular dystrophy
bp	base pair
BSA	bovine serum albumin
C	carboxyl
CaM	calmodulin
cDNA	complementary deoxyribonucleic acid
CFEOM	congenital fibrosis of the extraocular muscle
cfu	colony forming units
CMD	congenital muscular dystrophy
CNBr	cyanogen bromide
CNS	central nervous system
CR	cysteine rich region
CSD	cold shock domain
CT-1	carboxyl terminus of dystrophin consisting of amino acid number 3,485-3,685.
°C	degree(s) celcius

DAG	dystrophin associated glycoprotein
DAP	dystrophin associated protein
dbpA	DNA binding protein A
DG	dystroglycan
DGC	dystrophin glycoprotein complex
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DRP	dystrophin related protein
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
GST	glutathione S-transferase
HIS	histidine
hr	hour
IgG	immunoglobulin G
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton

KLH	keyhole limpet haemocyanin
Lac Z	lac Z reporter gene
LB	Luria-Bertani
LGMD	limb girdle muscular dystrophy
MAP	mitogen activated kinase
MCS	multiple cloning site
mRNA	messenger ribonucleic acid
ONPG	o-nitrophenyl β-D galactopyranoside
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl difluoride
psi	pounds per square inch
SCARMD	severe childhood autosomal recessive muscular dystrophy
SCE	sorbitol EDTA media
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SG	sarcoglycan
SL	sarcolemma
TBS	tris-buffered saline
TE	tris-EDTA
Tris	tris (hydroxymethyl) aminomethane
w/v	weight / volume

WWS	Walker-Warburg syndrome
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	yeast peptone media
YTA	yeast tryptone ampicillin media

Protein abbreviations:

A	alanine
C	cysteine
D	aspartate
E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine

W	tryptophan
Y	tyrosine

Greek symbols:

α	alpha
β	beta
γ	gamma
δ	delta

CHAPTER ONE

INTRODUCTION

1.1 Muscular Dystrophy.

Muscular dystrophy is a devastating and fatal disease, affecting 1 in every 3,300 male births (Emery, 1991). There are no obvious clinical symptoms until the age of 3 to 5 years (Emery, 1987). However, affected individuals can be diagnosed by histological analysis (Dubowitz, 1985), and identification of increased levels of serum creatine kinase (Rosalki, 1967). One of the first clinical observations of Muscular dystrophy is proximal muscle weakness and hypertrophy of calf muscles. Progressive loss of muscle strength results in loss of ambulation at about age 11 and confinement to a wheel chair. In addition to muscle pathology, mental retardation is present in about 30% of all Duchenne muscular dystrophy (DMD) patients (Moser, 1984). Early death usually occurs in the late teens or early twenties as a result of cardiomyopathy (Sakata *et al.*, 1990; De Vissier *et al.*, 1992; Nigro *et al.*, 1995; Fu *et al.*, 1997), pulmonary infection and/or respiratory failure (Vignos, 1977).

The most common and lethal of the Muscular dystrophies is Duchenne muscular dystrophy (DMD) first described by Meryon (1852) and Duchenne (1868). Becker muscular dystrophy (BMD) results in a milder phenotype (Koenig *et al.*, 1989). DMD and BMD are X-linked recessive disorders.

1.2 The Dystrophin Gene.

The gene responsible for DMD was mapped to the short arm of the X chromosome at Xp21 (Monaco *et al.*, 1986; Worton and Thompson, 1988). Dystrophin, the protein product of the DMD gene was identified in 1987 (Hoffman *et al.*, 1987). The gene coding for dystrophin is 2.3 million base pairs long and contains approximately 83

exons (Koenig *et al.*, 1987; Roberts *et al.*, 1992; Ahn and Kunkel, 1993). The DMD gene encodes a 14 kb mRNA and a 427 kDa dystrophin protein (Hoffman *et al.*, 1987).

Deletions, mutations or truncations of the DMD gene may result in lack of expression of dystrophin leading to DMD or in the expression of a truncated form of the protein resulting in BMD (Bulman *et al.*, 1991; Bushby *et al.*, 1993). Deletions of the gene occur with approximately equal frequency in DMD and BMD and deletion size does not correlate with the severity of the disease (Hart *et al.*, 1987; Liechti-Gallati *et al.*, 1987; Davies *et al.*, 1988; Lindlof *et al.*, 1988). Sixty-four percent of DMD patients are the result of intragenic deletions, compared to 75% of BMD patients (Simard *et al.*, 1992). Five percent of mutations in DMD and BMD are the result of duplications of the DMD gene (Hu *et al.*, 1990). Deletions identified in a number of DMD patients show a shift in the translational open reading frame (ORF) of triplet codons for amino acids, each deletion results in a truncated, abnormal dystrophin protein product (Monaco *et al.*, 1988).

A very mild form of BMD is seen in patients involving large in-frame deletions in the structural rod domain of dystrophin, this type of deletion shows little effect on protein stability, membrane binding or protein function (Gospe *et al.*, 1989; England *et al.*, 1990).

Attempts have been made at gene therapy for patients suffering from DMD, unfortunately to date none have been successful (Coovert and Burghes, 1994; Inui *et al.*, 1996). Adenovirus mediated gene transfer of a truncated dystrophin cDNA into a dystrophin deficient golden retriever dog model showed high levels of dystrophin expression in skeletal muscle (Howell *et al.*, 1998). There are many problems

encountered with gene therapy, mainly maintaining the levels of protein expression and counteracting rejection, leaving no known cure for the effects of muscular dystrophy.

1.3 The Dystrophin Protein.

The dystrophin protein is present in very low amounts in the cell and represents 0.002% of total muscle proteins (Hoffman *et al.*, 1987), and 5% of total membrane cytoskeletal proteins (Ohlendieck and Campbell, 1991a). The 427 kDa dystrophin protein is expressed in all muscle types and is located at the sarcolemma and the neuromuscular junctions in normal adult skeletal muscle fibers, smooth muscle fibers and cardiac muscle fibers. Expression of dystrophin is not restricted to muscle and has also been found in the brain, particularly in the cerebellum (Lidov *et al.*, 1990), glial and neuronal cells (Hoffman *et al.*, 1987), Purkinje cells (Huard and Tremblay, 1992) and in the peripheral nerves (Byers *et al.*, 1993).

The function of dystrophin is still not known, but it is widely accepted that the protein may play a structural role in skeletal muscle fibers, providing a link between the extracellular matrix and the cytoskeleton. This may be important in protecting muscle cells from the mechanical stress developed during muscle contraction (Petrof *et al.*, 1993; Pasternak *et al.*, 1995; Stevenson *et al.*, 1997).

1.3.1 Structural Domains of the Dystrophin Protein.

The dystrophin protein is comprised of four domains; (i) the N-terminus region, (ii) rod domain, (iii) cysteine rich region and (iv) the C-terminal region (see Figure 1-2).

(i) The N-terminus of dystrophin contains 240 amino acids and is the site for binding of F-actin (Hemmings *et al.*, 1992; Way *et al.*, 1992). The N-terminal domain has significant amino acid sequence similarity to the actin binding domain of α-actinin (Hammonds, 1987).

(ii) The rod domain of dystrophin is made up of 25 triple helical repeat sequences of 88-126 amino acid residues. Similar repeats are also found in spectrin and α-actinin (Davison and Critchley, 1988; Cross *et al.*, 1990). The repeats may promote α-helical coiled-coil interactions. Recently the rod domain was also shown to bind F-actin suggesting that actin may bind along-side the dystrophin molecule (Rybakova *et al.*, 1996).

(iii) The cysteine rich region of dystrophin includes amino acid residues 3,080-3,360. This region of the protein shows homology to the C-terminal end of the α-actinin from slime mold, and has also been shown to bind calcium *in vitro* (Milner *et al.*, 1992). The cysteine rich region contains two EF hand motifs, a ZZ domain which may bind zinc (Michalak *et al.*, 1996), a WW domain and two leucine heptad repeats. The WW domain is also the site of attachment for β-dystroglycan (Jung *et al.*, 1995).

(iv) The C-terminal region of dystrophin consists of the last 320 amino acids. This domain is unique and contains the most conserved amino acid sequences in the protein (Lemaire *et al.*, 1988; Bies *et al.*, 1992). There are many phosphorylation sites found at the C-terminus of dystrophin (Michalak *et al.*, 1996). This region of the protein is a substrate for endogenous protein kinases (Luise *et al.*, 1993; Madhaven and Jarrett, 1995) including cdc2 kinase (Milner *et al.*, 1993), and MAP kinase ($p44^{erk1}$ and $p42^{mpk}$) (Shemanko *et al.*, 1995). The C-terminal region of dystrophin is also phosphorylated by

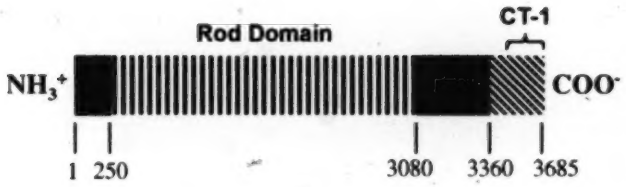
Casein kinase-2, Ca^{2+}/CaM-dependent kinase II (Madhavan and Jarrett, 1995; Walsh *et al.*, 1995; Michalak *et al.*, 1996), and dephosphorylated by calcineurin (Type 2B, Ca^{2+}/ CaM-dependent phosphatase) (Walsh *et al.*, 1995). There are several important observations suggesting that the C-terminal region of dystrophin may be one of the most important domains in the protein; (1) the high amino acid sequence conservation of this region; (2) a number of specific phosphorylation sites in this region suggesting its role in signal transduction from the extracellular matrix to the cytoskeleton; (3) this region, when deleted in DMD patients leads to the most severe phenotype.

1.4 The Dystrophin Isoforms.

Several isoforms of dystrophin have been identified (Ahn and Kunkel, 1993). There are at least seven distinct promoters located on the dystrophin gene which are responsible for cell type specific expression of different forms of dystrophin (Klamut *et al.*, 1989; Bar *et al.*, 1990; Barneau *et al.*, 1990; Chelly *et al.*, 1990; Boyce *et al.*, 1991; Makover *et al.*, 1991; Gorecki *et al.*, 1992). Three of these promoters are located at the 5' region of the dystrophin gene and each encode their own first exon giving rise to a predicted 427 kDa muscle type, brain type and Purkinje-cell-type isoform (Figure 1-1) (Fabbrizio *et al.*, 1994a). In addition there have been three internal promoters identified which control expression of 7.5, 5.5 and 4.8 kb mRNAs encoding short forms of dystrophin proteins, Dp140, Dp116 and Dp71 respectively (Figure 1-1) (Lederfein *et al.*, 1992; Byers *et al.*, 1993; Fabbrizio *et al.*, 1994a; Lidov *et al.*, 1995).

Lack of expression of the Dp140 dystrophin isoform is a result of a deletion in the DMD gene where the promoter region for Dp140 lies (Lidov *et al.*, 1995). Dp140 is

Figure 1-1. Schematic Representation of Dystrophin and the Dystrophin Isoforms. Schematic showing full length human skeletal muscle dystrophin consisting of four domains: the N-terminus (amino acid residues 1-240), the rod domain (amino acid residues 250-3,080), the cysteine rich region (amino acid residues 3,080-3,360) and the C-terminus (the last 325 amino acids). The CT-1 region (amino acids 3,484-3,685) is also shown. Four of the dystrophin isoforms are shown and depicted as Dp260, Dp140, Dp116 and Dp71. The cell type with the highest expression of a specific isoform is indicated. CR, cysteine rich region. The amino acid numbers are indicated for the dystrophin protein.



Dystrophin
Skeletal and Cardiac muscle, Brain and Cardiac Purkinje cells

Dp260
Retina

Dp140
Central nervous system

Dp116
Peripheral nerve

Dp71
Non-muscle

found throughout the central nervous system and a deletion of this kind could be a possible explanation for about one third of DMD patients suffering from mental retardation (Lidov *et al.*, 1995).

Dp116 is localized to the submembrane of the external layer of Schwann cells of the peripheral nerves (Byers *et al.*, 1993). Dp71 is expressed in a variety of non-muscle tissues, but is the most abundant dystrophin transcript found in the brain (Gorecki and Barnard, 1995). In addition to Dp71's expression in non muscle tissue, Fabbrizio *et al.* (1994b) showed localization of this dystrophin transcript to the transverse T tubules and in the Z-disk area associated with the dystrophin glycoprotein complex (DGC) in both bovine and chicken cardiac muscle.

Another isoform of dystrophin, named Dp260 containing the spectrin-like rod domain of full length dystrophin is expressed in retina and at a lower level in brain and cardiac tissue (D'Souza *et al.*, 1995). Dp260 is mainly localized to the outer plexiform layer of the retina, and is required for normal retinal function (D'Souza *et al.*, 1995). Interestingly, the Dp71 isoform and the full-length dystrophin transcript are also expressed in abundance in the retina and are localized to the outer plexiform layer. This would suggest that all three isoforms may play distinct functions within the same tissue. The three isoforms (Dp260, Dp71 and dystrophin) cannot function interchangeably (D'Souza *et al.*, 1995).

More evidence that the various dystrophin isoforms possess distinct functions is documented by studies on the transgenic *mdx* mouse, an animal model for DMD, lacking dystrophin (Greenberg *et al.*, 1994). The *mdx* mice express Dp71 and the protein is localized to the muscle plasma membrane (Greenberg *et al.*, 1994). Expression of Dp71

restores normal levels of dystrophin associated proteins (DAPs) indicating that Dp71 interacts with the DAPs in a similar manner to full length dystrophin. However, the restoration of the DAP complex was not sufficient to lessen the DMD pathology in *mdx* mice.

Another promoter in lymphoblastoid cells, located more than 500 kb upstream of the most 5' of all the identified promoters, was discovered in a patient suffering from DMD (Nishio *et al.*, 1994).

In addition to various isoforms of dystrophin, alternative splicing of the dystrophin transcript also occurs giving rise to variants in the C-terminal region of the protein (Feener *et al.*, 1989; Bies *et al.*, 1992).

1.5 Utrophin.

An autosomal homologue to dystrophin was discovered when Love *et al.* (1989), screened a fetal muscle cDNA library with cDNA clones encoding the C-terminal domain of Xp21 dystrophin and found a closely related sequence with 83% amino acid homology to dystrophin. This dystrophin related protein (DRP1), also known as utrophin, is translated from a 13 kb mRNA to give a 395 kDa protein (Tinsley *et al.*, 1993). In humans the utrophin gene is located at the 6q24 locus of chromosome 6 (Love *et al.*, 1989).

Utrophin (DRP1) is expressed mainly in nerve fibers, smooth muscle (especially that of the vascular system) and blood vessels but does not have the same pattern of distribution as dystrophin (Love *et al.*, 1993). Utrophin is part of the platelet membrane skeleton, and is thought to play a role in regulating the membrane stability and in the

distribution and function of adhesive receptors in transmembrane signaling (Earnest *et al.*, 1995). Unlike dystrophin, utrophin is expressed in the cytoplasm of cardiac Purkinje fibers and in intercalated disks but not at the cell periphery (Pons *et al.*, 1994a). In adult skeletal muscle, utrophin is restricted to the neuromuscular junctions where it colocalizes with acetylcholine receptors (Khurana *et al.*, 1991; Love *et al.*, 1991; Campanelli *et al.*, 1994). Interestingly, utrophin is overexpressed in skeletal muscle of DMD patients and appears to localize where dystrophin is deleted (Pons *et al.*, 1994b).

Amino acid sequence comparison between utrophin and dystrophin shows the overall domains to be very similar. Utrophin contains an actin binding domain in the first 250 amino acids, a rod domain consisting of 22 spectrin like repeats (Koenig *et al.*, 1988; Tinsley *et al.*, 1992; Pearce *et al.*, 1993), followed by a cysteine rich domain. The C-terminal region of utrophin is similar to dystrophin in that it binds the syntrophins (Kramarcy *et al.*, 1994)

Utrophin and dystrophin have many similarities. Utrophin is associated with the same complex of transmembrane glycoproteins as dystrophin (Matsumura *et al.*, 1992). Both dystrophin and utrophin are associated with the syntrophins (Kramarcy *et al.*, 1994) and both proteins bind actin (Hemmings *et al.*, 1992; Winder *et al.*, 1995).

Despite the observed similarities between utrophin and dystrophin, no disease has been linked to the absence of utrophin. The absence of utrophin may be lethal or may not result in clinical symptoms. However, the utrophin-null-*mdx* cross mouse, results in a very severe phenotype compared to the *mdx* mouse (Deconinck *et al.*, 1997; Grady *et al.*, 1997), so it is surprising that this phenotype has not been seen in humans, unless it is lethal.

Since utrophin shows high amino acid sequence homology to dystrophin and is known to bind to the DAPs, syntrophins, and actin it was suggested that utrophin could replace dystrophin at the sarcolemma in skeletal muscle cells and maybe even elleviate the symptoms of DMD and BMD. Gene transfer has been achieved by the introduction of utrophin mini-genes into the *mdx* mouse resulting in an improvement in muscle pathology and function, showing that utrophin may replace dystrophin at the sarcolemma (Tinsley *et al.*, 1996). The restoration of the DAPs to the sarcolemma seems to have been achieved by the successful expression of utrophin.

1.6 Dystrophin Related Protein 2.

Dystrophin related protein 2 (DRP2) is an isoform of utrophin. DRP2 is a relatively small protein, encoded by a 45 kb gene localized to Xq22. It is expressed mainly in the brain and spinal cord, with major sites of expression in the olfactory bulb, the outer layer of the cerebral cortex, the medial habenula, and the hippocampus (Roberts *et al.*, 1996). It is similar to the overall structure of the Dp116 dystrophin isoform (Roberts *et al.*, 1996). The protein sequence consists of a short N-terminal region, followed by sequences homologous to the last two spectrin-like repeats and the C-terminal domains of dystrophin and utrophin (DRP1).

1.7 The Dystrophin Glycoprotein Complex.

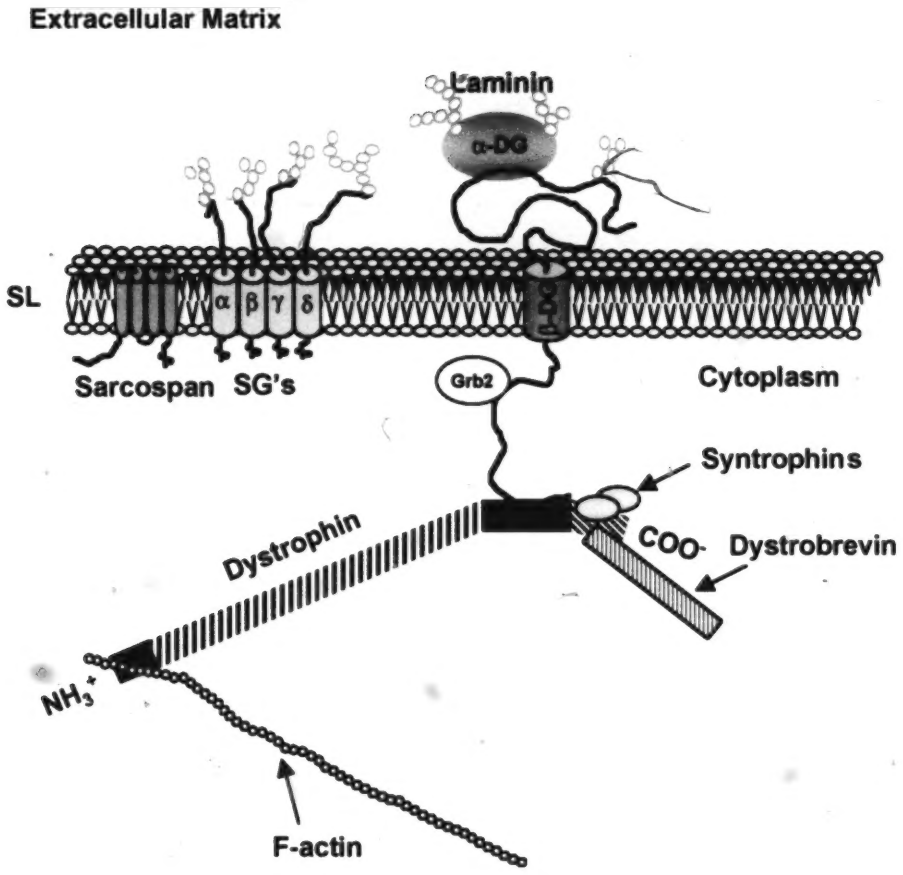
In mammalian skeletal muscle, dystrophin is found associated with several integral and peripheral membrane proteins (DAPs), forming a complex known as the dystrophin glycoprotein complex (DGC) (see Figure 1-2) (Ervasti and Campbell, 1991;

Ibraghimov-Beskrovnaya *et al*., 1992). The DGC is divided into three distinct entities; dystroglycans, sarcoglycans and syntrophins (Blake *et al*., 1996; Henry and Campbell, 1996; Michalak and Opas, 1997). Tight association of the DGC with dystrophin has been shown by their co-purification from rabbit skeletal muscle fiber membrane, co-sedimentation, immunoprecipitation, co-localization to muscle sarcolemma, and by protein cross-linking experiments (Ervasti and Campbell, 1991). The native molecular weight of purified DGC is large enough to contain one copy of each protein in the complex, including dystrophin (Rybakova and Ervasti, 1997).

Defects in dystrophin and / or other components of the DGC are responsible for several phenotypes of muscular dystrophy, including DMD, BMD and limb-girdle muscular dystrophy (LGMD) (Campbell, 1995a; Worton, 1995). Immunoblot analysis using total muscle membranes from control and *mdx* mice of ages 1 day to 30 weeks indicates that all of the DAPs are greatly reduced (80-90%) in skeletal muscle of *mdx* mouse (Ohlendieck and Campbell, 1991b). In BMD there is a small reduction of the DAPs, consistent with a reduced content of dystrophin. The absence of dystrophin or the lack of its cysteine rich or C-domain results in the absence of all the components of the DAPs in the muscle sarcolemma.

Figure 1-2. The Dystrophin Glycoprotein Complex.

This is a model of the dystrophin glycoprotein complex (DGC) prior to commencing my project. The DGC is located at the sarcolemma of a skeletal muscle cell. The DGC is composed of dystrophin, dystroglycans (α- and β-dystroglycan), sarcoglycans (α-SG, β-SG, γ-SG, and δ-SG) and sarcospan. A more detailed description can be found under the "Introduction".


Extracellular Matrix
Laminin
α-DG
SL
α
β
γ
δ
β-DG
Sarcospan
SG's
Grb2
Cytoplasm
Syntrophins
Dystrophin
COO-
Dystrobrevin
NH3+
F-actin

1.7.1 The Dystroglycans.

Both α- and β-dystroglycan are encoded by the same 5.8 kb mRNA. Post-translational modification of a 97 kDa precursor protein results in two mature proteins, a 43 kDa and a 156 kDa DAP (Ibraghimov-Beskrovnaya *et al.*, 1992). The amino acid sequence of human and rabbit dystroglycan are 93% identical (Ibraghimov-Beskrovnaya *et al.*, 1993).

α-Dystroglycan is an extracellular peripheral membrane protein that binds to merosin, a laminin subunit, in the basement membrane (Ibraghimov-Beskrovnaya *et al.*, 1992). β-Dystroglycan is an integral transmembrane protein that interacts with the cysteine rich domain of dystrophin (Suzuki *et al.*, 1992).

β-Dystroglycan also interacts with Grb2, an adapter protein involved in signal transduction and cytoskeletal organization (Yang *et al.*, 1995). The Grb2-dystroglycan interaction may play an important role in extracellular matrix-mediated signaling. One major function of the dystroglycans is to link the extracellular matrix to the cell cytoskeleton (Ibraghimov-Beskrovnaya *et al.*, 1992; Ervasti and Campbell, 1993). Recently, skeletal muscle from a patient suffering from DMD like symptoms showed a selective deficiency of β-dystroglycan with normal expression of α-dystroglycan, which has not been reported in any other type of neuromuscular disorder (Salih *et al.*, 1996). The lack of β-dystroglycan (Salih *et al.*, 1996) may result in a loss of communication and structural support between the intracellular cytoskeleton (dystrophin) and the extracellular matrix (laminin, via α-dystroglycan).

1.7.2 The Sarcoglycans.

The sarcoglycan (SG) complex is made up of four integral membrane proteins;α-, β-, γ-, and δ-SGs. All four SGs have a small intracellular domain composed of either the C-terminus (α-SG) or the N-terminus (β-, γ-, and δ-SGs), one transmembrane domain and a large extracellular region with predicted N-glycosylation residues (Ozawa *et al.*, 1998).

The loss of any of α-, β-, γ-, or δ-SGs is responsible for autosomal recessive limb girdle muscular dystrophy (LGMD-2D, LGMD-2E, LGMD-2C or LGMD-2F respectively), with a phenotype similar to DMD (Worton *et al.*, 1995). In patients with a loss in α-SG the disease pathology is particularly severe and is referred to as severe childhood autosomal recessive muscular dystrophy or SCARMD (Ben Hamida *et al.*, 1983; Ben Jelloun-Dellagi *et al.*, 1990). Muscle weakness and atrophy of the proximal and pelvic muscles are the typical features of SCARMD and effects females as well as males with equal frequency (Campbell, 1995b). In North Africa, the incidence of SCARMD, accounts for 10 to 50% of total muscular dystrophy cases (Ben Hamida *et al.*, 1992).

1.7.2.1 α-Sarcoglycan.

α-SG (previously called adhalin or 50 DAG) was first identified in rabbit skeletal muscle in 1980, and given the name SL50 for its apparent molecular weight of 50,000 (Jorgensen *et al.*, 1980). Association of α-SG with the DGC in skeletal muscle was reported in 1990 (Ervasti *et al.*, 1990; Yoshida *et al.*, 1990).

The gene for α-SG maps to chromosome 17 (Roberds *et al.*, 1994). Patients suffering from autosomal recessive limb girdle muscular dystrophy have a missense mutation in both alleles of this gene (Roberds *et al.*, 1994). It has been suggested that less than 5% of DMD patients have defects in the α-SG gene (Ljunggren *et al.*, 1995). However, this may not be an accurate estimate, since many DMD patients might be misdiagnosed due to similar clinical symptoms.

Complete cloning of α-SG cDNA was reported by two groups, Roberds *et al.* (1994), and McNally *et al.* (1994). The expression of a 1.5 kb α-SG mRNA is restricted to skeletal, cardiac, and selected smooth muscle (Roberds *et al.*, 1993; Yamamoto *et al.*, 1994). The α-SG protein is expressed at approximately 6 fold higher levels in skeletal muscle than in cardiac muscle (Roberds *et al.*, 1993).

The open reading frame of α-SG encodes a 387 amino acid protein (McNally *et al.*, 1994). Human and rabbit α-SG proteins share 90% homology at the amino acid level and have a hydrophobic signal sequence, a single transmembrane domain, two potential N-linked glycosylation sites in the putative extracellular domain and one consensus site for phosphorylation by Ca^{2+}/CaM-dependent protein kinase (McNally *et al.*, 1994).

Several different mutations of the α-SG gene including missense, nonsense, splicing, and in-frame deletions have been reported in SCARMD patients (Roberds *et al.*, 1994; Kawai *et al.*, 1995; Ljunggren *et al.*, 1995; Passos-Bueno *et al.*, 1995; Piccolo *et al.*, 1995; Fadic, *et al.*, 1996; Quinlivan *et al.*, 1997). Severe disease phenotypes were observed in patients with null α-SG mutations, whereas milder phenotypes were found in patients with missense mutations in the α-SG gene (Piccolo *et al.*, 1995; Fardeau *et al.*, 1996).

In two cases of Walker-Warburg syndrome, the skeletal muscle was severely deficient in the laminin β2 chain and also in α-SG (Wewer *et al.*, 1995). Walker-Warburg syndrome (WWS) is an autosomal recessive disorder characterized by cerebellar and ocular malformations and muscular dystrophy (Dobyns *et al.*, 1989; Miller *et al.*, 1991). The gene for WWS was mapped to chromosome 9q31-33 (Toda *et al.*, 1995), suggesting that an α-SG mutation is not the primary cause of WWS. However, these studies indicate that the interaction of laminin chains and α-SG play an important role in muscle function, since the loss of α-SG is secondary to the loss of laminin.

Abnormal expression of the β1 laminin subunit was also observed in two Japanese patients suffering from SCARMD, there was a marked reduction in the levels of β1 laminin expression in the basal lamina surrounding muscle fibers (Higuchi *et al.*, 1994). These results indicate that a deficiency of α-SG may be associated with the disturbance of sarcolemma-extracellular matrix interactions, and the loss of α-SG may lead to sarcolemma instability.

1.7.2.2 β-, γ-, and δ-Sarcoglycans.

The β-SG gene has been mapped to chromosome 4q12 (Bonnemann *et al.*, 1995; Lim *et al.*, 1995). A defective β-SG gene results in LGMD-2E (Bonnemann *et al.*, 1995; Lim *et al.*, 1995). The predicted 43 kDa glycosylated transmembrane β-SG, is highly conserved between rabbit and human (Bonnemann *et al.*, 1995). β-SG mRNA is most abundantly expressed in striated muscle, but is also found in lower levels in brain,

kidney, placenta, pancreas, and lung. This is unlike α-, γ-, and δ-SG proteins which are limited to expression in muscle.

γ-SG has been mapped to chromosome 13q12 (Noguchi *et al.*, 1995). It is a 35 kDa integral membrane protein (Noguchi *et al.*, 1995). The predicted amino acid sequences of γ-SG is highly conserved between human and rabbit (89% amino acid identity and 93% similarity) (Noguchi *et al.*, 1995). The cytoplasmic domain of γ-SG contains a potential phosphorylation site for Casein kinase II, and the extracellular domain contains one conserved asparagine-linked glycosylation site (Noguchi *et al.*, 1995). The human γ-SG mRNA is expressed exclusively in striated muscle, both cardiac and skeletal (Noguchi *et al.*, 1995).

Deletions in the γ-SG gene have been identified in a number of families (Jung *et al.*, 1996a, McNally *et al.*, 1996a and 1996b). For example a deletion of a single base pair has been found in this gene in Tunisian LGMD patients (Noguchi *et al.*, 1995). These patients also showed a marked reduction in the α-, β- and δ-SGs, but other components of the DGC, dystrophin and syntrophin are unchanged (Jung *et al.*, 1996a; McNally *et al.*, 1996a and 1996b; Li *et al.*, 1998).

Mice lacking γ-SG develop muscular dystrophy early in life which affected the proximal skeletal muscles, similar to LGMD in humans (Hack *et al.*, 1998). Mice lacking γ-SG also develop cardiomyopathy affecting both the left and right ventricle (Hack *et al.*, 1998). Interestingly, dystrophin, dystroglycan, and laminin are normally expressed at the sarcolemma in these mice.

The δ-SG gene is localized to chromosome 5q33-34 (Passos-Bueno *et al.*, 1996). δ-SG is highly expressed in both skeletal and cardiac muscle (Jung *et al.*, 1996b). A

single nucleotide deletion in the δ-SG gene, that alters its reading frame, is the cause of LGMD-2F (Nigro *et al.*, 1996). In LGMD-2F there is also a reduction in the expression of the other SGs (Jung *et al.*, 1996b).

Recently it was shown that SG gene transfer using a recombinant δ-SG adenovirus into a BIO 14.6 hamster (which is a model for SG deficient LGMD), could successfully up-regulate the other proteins in the SG complex and show long term expression of δ-SG (Holt *et al.*, 1998).

1.7.3 Syntrophins.

The syntrophin complex consists of α-, β1- and β2-syntrophins, which bind to the C-terminal region of dystrophin. The three syntrophin proteins share 50-60% identity in their amino acid sequences. In skeletal muscle dystrophin and syntrophin are present at the peripheral sarcolemma and neuromuscular junction, whereas in cardiac muscle they are associated with the peripheral sarcolemma and T-tubule membranes (Iwata *et al.*, 1998).

The α-syntrophin is expressed in skeletal and cardiac muscle (Adams *et al.*, 1993). The β1- and β2- syntrophins are expressed in various tissues, with the exception that β1-syntrophin is expressed in relatively low amounts in brain and heart (Ahn *et al.*, 1996). Different members of the dystrophin family interact with different syntrophin complexes likely due to the different tissue distribution patterns of syntrophins. Utrophin is complexed with β1- and β2-syntrophins while dystrophin associates with α1- and β2-syntrophins (Ahn *et al.*, 1996). Dystrobrevin complexes contain dystrophin and α1- and β1-syntrophins (Peters *et al.*, 1997a).

1.7.4 Sarcospan.

Sarcospan is a 25 kDa integral membrane protein which co-purifies with the DGC (Crosbie *et al.*, 1997). The protein is expressed in skeletal muscle and the heart (Crosbie *et al.*, 1997). Predicted topology for sarcospan indicates that the protein contains four transmembrane spanning domains (Crosbie *et al.*, 1997), unlike the single transmembrane domains present in SG proteins. The N and C-terminus are located intracellularly (Crosbie *et al.*, 1997). The gene encoding sarcospan is located on human chromosome 12p11.2 (Heighway *et al.*, 1996), the locus for congenital fibrosis of the extraocular muscle (Engle *et al.*, 1994; Engle *et al.*, 1995). Congenital fibrosis of the extraocular muscle (CFEOM) is an autosomal dominant disorder, which effects the ocular muscles leaving patients with little or no eye movement and sarcospan is thought to be defective in CFEOM (Engle *et al.*, 1994; Engle *et al.*, 1995). It is not known if there is a reduction in the expression of any other DGC proteins in patients suffering from this condition.

1.8 Dystrobrevin.

α- and β-dystrobrevins are likely to associate directly with dystrophin (Suzuki *et al.*, 1995). Dystrobrevin was first described as a tyrosine-phosphorylated 87-kDa protein in *Torpedo* electric organ (Carr *et al.*, 1989; Wagner *et al.*, 1993). *Torpedo* dystrobrevin is composed of a cysteine rich region with limited homology to dystrophin and a unique C-terminal tail, containing tyrosine phosphorylation sites (Blake *et al.*, 1996). The remaining amino acid residues of α- and β-dystrobrevins are highly related to each other but not to dystrophin, utrophin or DRP2 (Peters *et al.*, 1997b).

Two isoforms of α-dystrobrevin are generated by alternative splicing and are both expressed in skeletal muscle (Blake *et al.*, 1996; Sadoulet-Puccio *et al.*, 1996). α-Dystrobrevin-1, which contains the C-terminal extension and α-dystrobrevin-2, which lacks this extension (Blake *et al.*, 1996; Sadoulet-Puccio *et al.*, 1996). α-Dystrobrevin-2 is localized to the neuromuscular junction and co-purifies with dystrophin from muscle extracts (Peters *et al.*, 1997b). α-Dystrobrevin-1, however, co-purifies with utrophin and is expressed in the synpase (Peters *et al.*, 1998).

Interestingly, the WW domain is missing in the dystrobrevins, this region is responsible for the interaction of dystrophin with β-dystroglycan (Jung *et al.*, 1995). Therefore, the function of the dystrobrevins in the cytoskeleton is different to that of dystrophin or utrophin and it is likely that the dystrobrevins may not interact directly with the DAPs.

In common with other dystrophin-associated proteins, the level of dystrobrevin at the sarcolemma of DMD patients is dramatically reduced (Metzinger *et al.*, 1997).

1.9 Laminin.

Laminin is one of the main components of the basal lamina (Ibraghimov-Beskrovnaya *et al.*, 1992). The protein binds to α-dystroglycan which interacts with β-dystroglycan (Ibraghimov-Beskrovnaya *et al.*, 1992). Therefore, β-Dystroglycan may be considered the link between dystrophin and laminin. The absence of laminin is enough to severely disrupt this extracellular link with the cytoskeleton and results in a muscular dystrophy pathology known as Congenital muscular dystrophy (Tome *et al.*, 1994). CMD is caused by the deficiency in the laminin α2 chain (Tome *et al.*, 1994). CMD

pathology is evident at birth or in the first few months of life and consists of muscle weakness and hypotonia, severe and early contractures and sometimes joint deformities (Tome *et al.*, 1994).

1.10 A Defective Dystrophin Glycoprotein Complex.

It is interesting to see that a defect in the laminin subunit, the sarcoglycans, sarcospan or the dystroglycans leads to various forms of muscular dystrophy. All of these muscular dystrophies are independent of defects in dystrophin. It seems that all the proteins in the DGC are equally important in maintaining the sarcolemma membrane stability. Furthermore, they may play a role in transducing signals from the extracellular matrix to the cytoskeleton. The proteins of the DGC seem to act in conjunction with one another, which may explain the loss of the DAPs when one protein is missing or when dystrophin is absent. However, it is still not clear if the DAPs are the only proteins involved in the structural and functional and communication-link between dystrophin the cytoskeletal proteins and the extracellular matrix.

1.11 Dystrophin and Cytoskeletal Proteins.

Costameres are structures linking the sarcolemma to Z-lines in striated muscle (Pardo *et al.*, 1983). Dystrophin co-localizes at the costameres with other cytoskeletal proteins including vinculin, α-actinin, talin, ankyrin, F-actin, and β-spectrin (Minetti *et al.*, 1992; Straub, *et al.*, 1992). Costameres are believed to play roles in cell adhesion (Berthier and Blaineau, 1997). Cell adhesion complexes consist of transmembrane receptors which anchor intracellular cytoskeletal proteins and signal transduction

molecules. The transmembrane receptor, integrin is one of these molecules (Berthier and Blaineau, 1997). Integrins are known to bind talin and α-actinin (Berthier and Blaineau, 1997). Talin in turn binds to vinculin and actin (Yamada and Geiger, 1997) leading to the formation of the integrin-talin-actinin-vinculin-actin complex. Dystrophin may also be a component of the integrin/cytoskeleton complex (Yoshida *et al.*, 1998) but its functional significance is not clear. In the *mdx* mouse, expression of both vinculin and talin in the myotendinous junctions of skeletal muscle increases by approximately 200% (Law *et al.*, 1994). Since vinculin and talin are components of the protein complex which anchors actin to the cell membrane, an increase in vinculin and talin expression may be considered a compensation reaction for the loss of dystrophin. These adhesion proteins, therefore, might play an additional role in anchoring dystrophin to the sarcolemma (Senter *et al.*, 1993).

In summary dystrophin plays both a structural and signaling role in the cell cytoskeleton, which is vital for the viability of skeletal muscle cells. Exactly how these processes are carried out and which molecules are involved requires further studies.

Objectives.

The structural and functional role of dystrophin and components of the dystrophin glycoprotein complex in the sarcolemma of muscle cells is not fully understood. The focus of my studies was to identify new components of the DGC, with a special emphasis on molecules associated with the C-terminus of α-sarcoglycan and the C-terminus of dystrophin. These are both extremely important regions of these proteins in the pathology of muscular dystrophy. These protein-protein interactions may play a key role in the pathology of DMD and SCARMD and contribute to muscle cell function. The specific objectives of my research project were:

(1) To investigate and identify proteins interacting with the C-terminus of α-sarcoglycan.

(2) To identify new proteins associated with the unique C-terminal region of dystrophin.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

The yeast-two hybrid skeletal muscle cDNA library, expression vectors, the *E.coli* strains HB101 and DH5α and the yeast strain HF7c were obtained from Clontech Laboratories. Tris, Triton X-100, Lyticase, Nycodenz, wheat germ lectin, IPTG, N-acetyl-D-glucosamine, formaldehyde, DTT, ONPG, Freunds complete and incomplete adjuvant, PEG, Bromophenol Blue and α-actin antibodies were purchased from Sigma. β-mercaptoethanol was from BDH. Whatmann #5 paper was purchased from Fisher Scientific. Immunopure Gentle Elution Ag/Ab buffer was purchased from Pierce. Peroxidase-conjugated goat anti-rabbit IgGs were from Boehringer Mannheim. Nitrocellulose filters were from Millipore. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and molecular weight markers were from Bio-Rad. Benzamidine, phenylmethylsulfonyl fluoride, N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, aprotinin, leupeptin, pepstatin, L-1-Chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, L-Chloro-3-(4-tosylamido)-4-phenyl-2-butanone, (4-Amidinophenyl)-methanesulfonyl fluoride and phosphoramidone were obtained from Boehringer Mannheim. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) was purchased from Rose Scientific Ltd. Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were obtained from Gibco/BRL and/or Hyclone. X-ray film was from Kodak. Mono Q FPLC column was from Pharmacia. Activated CNBr Sepharose beads and Glutathione-Sepharose were purchased from Pharmacia Biotechnologies. Dystrophin cDNA was obtained from the American Type Culture Collection. Synthetic oligodeoxynucleotides were synthesized using an Applied Biosystems 392 DNA synthesizer in the DNA Core Facility Laboratory,

Department of Biochemistry, University of Alberta. Restriction endonucleases, DNA modifying enzymes, and Vent DNA polymerase were from Boehringer-Mannheim, Bethesda Research Laboratories and/or Gibco. All enzymes were used in buffers provided by the manufacturer. The *E.coli* strain BNN103 was a gift from Dr. J. Weiner of the University of Alberta. Peptides for antibody production were synthesized by the Alberta Peptide Institute, University of Alberta. The cDNA for full length α-actinin was a generous gift from Dr. A. H. Beggs, Harvard Medical School, Boston, Massachusetts. C_2C_{12} muscle cells were a gift from Dr. P. Holland, Montreal Neurological Institute, Quebec. All chemicals were of the highest grade available.

2.2 Cloning Procedures.

The polymerase chain reaction (PCR) was performed in ThermoPol reaction buffer containing 10 mM KCl, 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100 (Innis *et al.*, 1990; Ausubel *et al.*, 1996). The reaction mixture also included 5 mM of each dNTP, 100 ng of template, 200 ng of each primer and 1 unit of Vent DNA polymerase. The PCR products were concentrated by spin filtering in Millipore (Ultrafree-MC 30,000 NMWL Filter Units) spin-filter tubes.

Restriction endonuclease digestion of DNA was performed using the protocols recommended by the manufacturer. Ligations of plasmid and DNA were carried out at a molar ratio of Insert:Vector of 3:1, 0.1 units of T4 DNA ligase in the presence of T4 DNA ligase buffer (250 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% (w/v) polyethylene glycol-8000). The DNA ligation reaction was performed at 16°C for 16 hr.

DNA were transformed into DH5α competent cells unless stated otherwise. Fifty µl of competent cells were added to the ligation reaction and incubated on ice for 30 min, heat shocked for 2 min at 37°C, followed by 2 min incubation at 4°C. One ml of LB (Luria-Bertani media containing 1 % bacto-tryptone (w/v), 0.5 % bacto-yeast extract (w/v), and 1 % NaCl (w/v), pH 7.0) was added to cells and incubated for 30 min at 37°C with constant shaking. Cells were spun down and plated on selective agar plates. Small scale isolation of plasmid DNA was performed by the alkaline lysis method (Sambrook *et al.*, 1989). For large scale DNA preparations, alkaline lysis followed by PEG / NaCl precipitation was used (Sambrook *et al.*, 1989). In the Matchmaker yeast-two hybrid system, DNA was isolated by Qiagen column chromatography.

2.3 Construction of Plasmid DNA for the Yeast-2-Hybrid System.

PCR was used to synthesize the cDNA encoding the carboxyl 76 amino acids of human α-sarcoglycan (designated αSG-C; amino acids 312-387) (Figure 2-1). DNA from a human skeletal muscle cDNA library was used as the template (Clontech Laboratories). The primers SG-1 and SG-2 (Table 2-1) were used for the synthesis. The PCR product was cloned into the pGBT9 vector at EcoR1 and BamH1 restriction endonuclease sites, in-frame with the GAL4-DNA-binding domain. This clone was designated pGBT9-αSG-C (Figure 2-2).

The cDNA encoding the C-terminal region of dystrophin, corresponding to the last 200 amino acids of human dystrophin (designated CT-1; amino acids 3,485-3,685) (Milner *et al.*, 1992) was obtained by PCR amplification. The PCR reaction was carried out using a human dystrophin cDNA as template and the primers CT-A and CT-B (Table

Figure 2-1. Amino Acid Sequence and Predicted Topology of α-Sarcoglycan.

A, amino acid sequence of human α-sarcoglycan protein (amino acid residues 1-387). The amino acid sequence in the blue box indicates the predicted membrane spanning region. The underlined sequence are the amino acids which are located in the cytoplasm (amino acid residues 312-387). This region was designated as the C-terminus of α-sarcoglycan and was used for cloning and expression studies. The amino acid sequence in the pink box indicates the peptide used to generate antibodies to α-sarcoglycan (amino acid residues 376-387).

B, Diagrammatic representation of the predicted membrane topology of the α-sarcoglycan protein. The black line indicates the sequence at the extracellular side of the sarcolemma membrane. The blue cylinder indicates the transmembrane spanning region. The C-terminus is shown as individual amino acids, the amino acids in the pink circles indicate the peptide used to generate antibodies. This diagram is not to scale.

A

NH_3^+-MAETLFWTPLLVVLAGLGDTEAQQTTLHPLVGRVFVHTLDHETFLSLPEHVAVP
PAVHITYHAHLQGHPDLPRWLRYTQRSPHHPGFLYGSATPEDRGLQVIEVTAY
NRDSFDTTRQRLVLEIGDPEGPLLPYQAEFLVRSHDAEEVLPSTPASRFLSALG
GLWEPGELQLLNVTSALDRGGRVPLPIEGRKEGVYIKVGSASPFSTCLKMVASP
DSHARCAQGQPPLLSCYDTLAPHFRVDWCNVTLKVDKSVPEPADEVPTPGDGI
LEHDPFFCPPTEAPDRDFLVDALVTLLVPLLVALLLTLLAYVMCCRREGRLKRDL
ATSDIQMVHHCTIHGNTEELRQMAASREVPRPLSTLPMFNVHTGERLPPRVDSA
QVPLILDQH-COO-

B

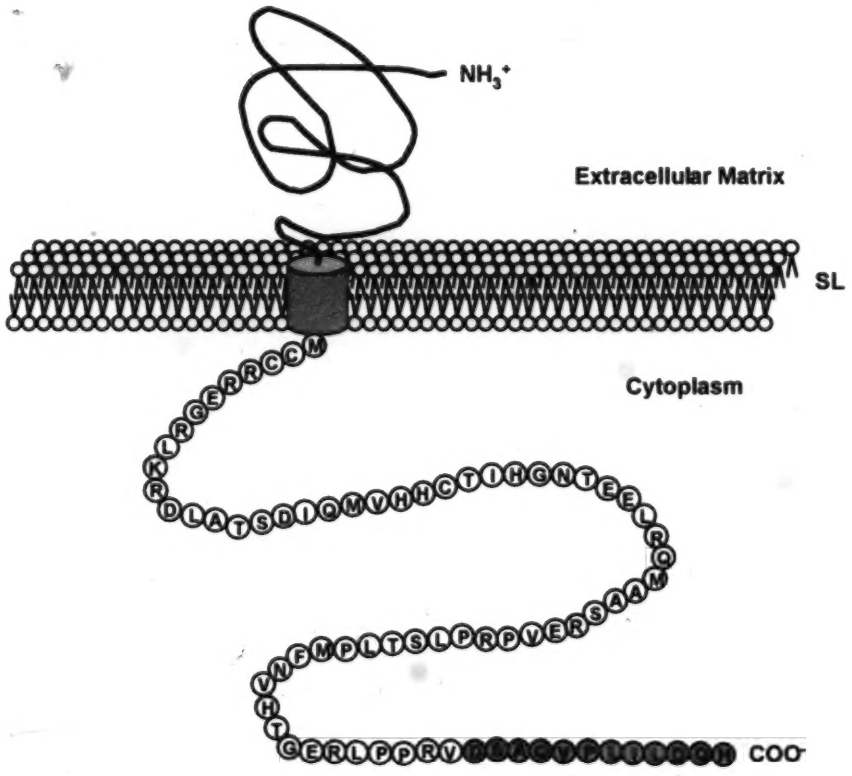


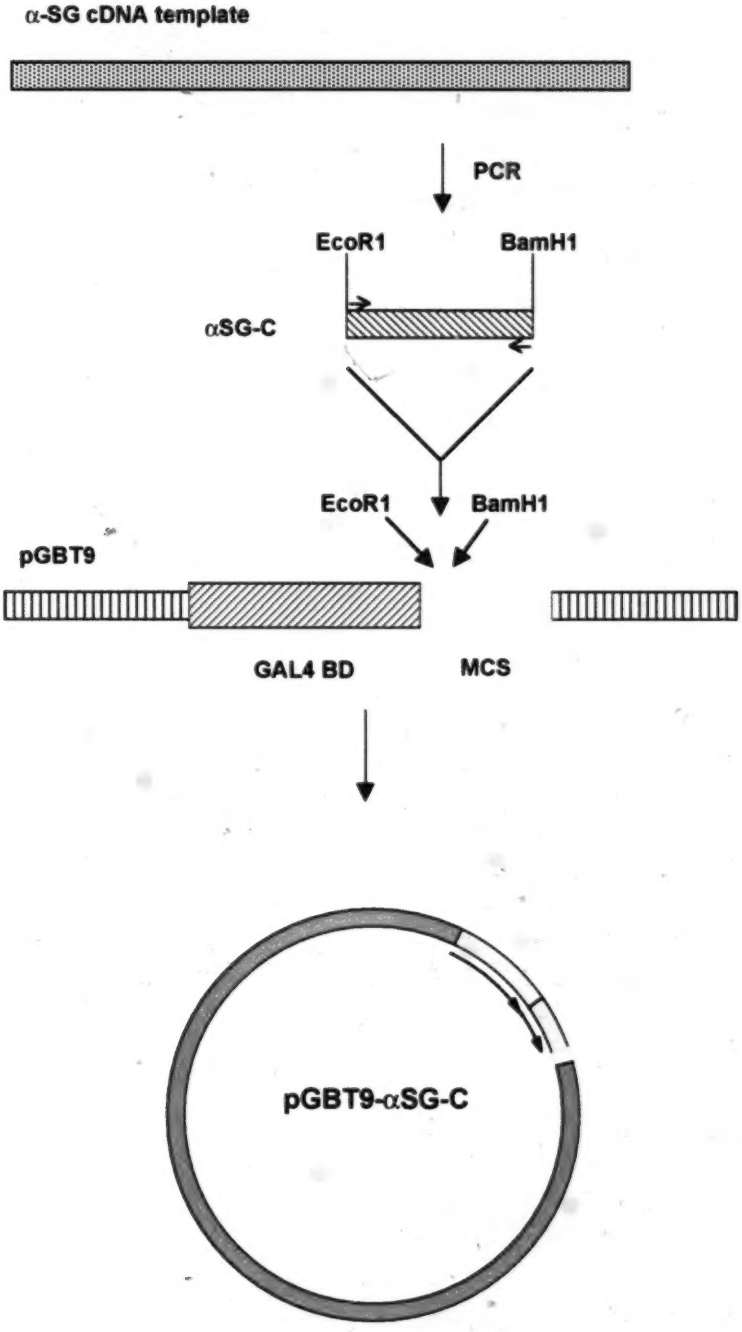
Table 2-1. DNA Primers used for the Generation of the Yeast Two-Hybrid and GST-fusion DNA Constructs.

NAME	NUCLEOTIDE SEQUENCE	NUCLEOTIDE SEQUENCE #
SG-1	5'-atatgaattcATGTGCTGCCGGCGG-3'	977-991 of human α-SG
SG-2	5'-atatggatccGGAACCACTGGCTGGGC-3'	1,210-1,226 of human α-SG
SG-3	5'-tataggatccCCATGTGCTGCCGGCGG-3'	977-991 of human α-SG
SG-4	5'-atatgaattcGGAACCACTGGCTGGGCTGTC-3'	1,206-1,226 of human α-SG
CT-A	5'-tatatggaattcCTGAGCCAGCCTCGTAGTCCT-3'	10,661-10,681 of human dystrophin
CT-B	5'-tgggatccCTAAGGACTCCATCGCTCTGC-3'	11,297-11,317 of human dystrophin
AC-1	5'-atatgaatccCAGATCTTGCTGCAGAA-3'	1,437-1,452 of human α-actinin
AC-2	5'-atagaatccCAGATCGCTCTCCCC-3'	2,841-2,855 of human α-actinin
AC-3	5'-atatgaattcCAGATCTTGCTGCAGAA-3'	1,437-1,452 of human α-actinin
AC-4	5'-atatggatccTCTCTCTAGGGCTTCTC-3'	1,675-1,691 of human α-actinin
AC-5	5'-cgcgaattcATGGAGAAATTGCTA-3'	1,692-1,776 of human α-actinin
AC-6	5'-atagaatccCAGATCGCTCTCCCC-3'	2,841-2,855 of human α-actinin

Underlined nucleotides represent endonuclease restriction sites.

Figure 2-2. Diagrammatic Representation of the Construction of pGBT9-αSG-C Plasmid.

The cDNA encoding αSG-C (nucleotides 977-1,226) was PCR amplified using a human skeletal muscle cDNA library as template and the primers SG-1 and SG-2 (Table 2-1) as indicated by the arrows. The PCR product (blue hatched rectangle) was then inserted into the pGBT9 vector in-frame with the GAL4 DNA binding domain (green hatched rectangle) at EcoR1 and BamH1 restriction endonuclease sites. The resulting plasmid was designated pGBT9-αSG-C. The arrows shown in the circular plasmid indicate the translated protein sequences. MCS: multiple cloning site.


α-SG cDNA template
PCR
EcoR1
BamH1
αSG-C
EcoR1
BamH1
pGBT9
GAL4 BD
MCS
pGBT9-αSG-C

2-1). The PCR product was cloned in-frame with the GAL4 DNA-binding domain at EcoR1 and BamH1 restriction endonuclease sites to generate the clone pGBT9-CT-1 (Figure 2-3).

The cDNA encoding the C-terminus of human skeletal muscle α-actinin-2 was amplified using the full length cDNA of α-actinin as template and the primers AC-1 and AC-2 (Table 2-1). The C-terminus of α-actinin-2 was then cloned in frame with the GAL4-DNA activation domain vector, pGAD424 and designated pGAD-α-Actinin-C (Figure 2-4).

The cDNA encoding two fragments of the C-terminus of α-actinin-2 were constructed (see Figure 4-2). Both cDNAs were amplified using the pGAD-α-Actinin-C vector as template. The first DNA fragment named α-actinin-C1 (nucleotide number 1,437-1,691) was amplified using the primers AC-3 and AC-4 (Table 2-1). The PCR product was cloned in frame with the GAL4-DNA activation domain to generate the clone pGAD-α-Actinin-C1 (Figure 2-5). The second cDNA fragment, named α-actinin-C2 (nucleotide number 1,692-2,855), was amplified using the primers AC-5 and AC-6 (Table 2-1). The PCR product was cloned in frame with the GAL4-DNA activation domain to generate the clone pGAD-α-Actinin-C2 (Figure 2-5).

2.4 The Matchmaker Yeast Two-Hybrid System.

2.4.1 cDNA Library Titering.

A human skeletal muscle cDNA library was obtained from Clontech Laboratories. The source of the mRNA was from the skeletal thigh muscle of a 30 year old Caucasian male. The human skeletal muscle cDNA library was constructed into a GAL4 activation

Figure 2-3. Diagrammatic Representation of the Construction of pGBT9-CT-1 Plasmid.

The cDNA encoding CT-1 (nucleotides 10,661-11,317) was PCR amplified using dystrophin cDNA as a template and the primers CT-A and CT-B (Table 2-1) as indicated by the arrows. The PCR product (blue hatched rectangle) was then inserted into the pGBT9 vector in-frame with the GAL4 DNA binding domain (green hatched rectangle) at EcoR1 and BamH1 restriction endonuclease sites. The resulting plasmid was designated pGBT9-CT-1. The arrows shown in the circular plasmid indicate the translated protein sequences. MCS: multiple cloning site.

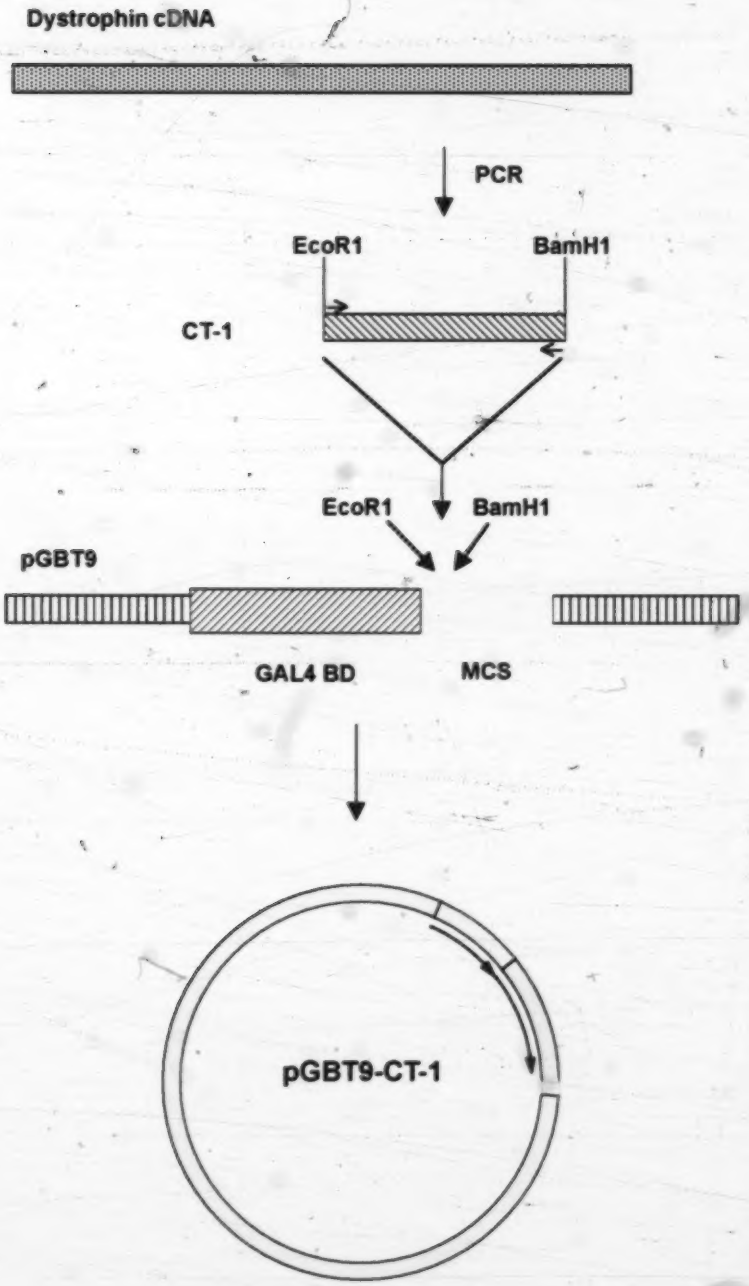

Dystrophin cDNA
PCR
EcoR1
BamH1
CT-1
EcoR1
BamH1
pGBT9
GAL4 BD
MCS
pGBT9-CT-1

Figure 2-4. Diagrammatic Representation of the Construction of pGAD-α-Actinin-C Plasmid.

The cDNA encoding α-actinin-C (nucleotides 1,437-2,855) was PCR amplified using full length α-actinin cDNA as template and the primers AC-1 and AC-2 (Table 2-1) as indicated by the arrows. The PCR product (blue hatched rectangle) was then inserted into the pGAD424 vector in-frame with the GAL4 DNA activation domain (green hatched rectangle) at EcoR1 restriction endonuclease sites. The resulting plasmid was designated pGAD-α-Actinin-C. The arrows shown in the circular plasmid indicate the translated protein sequences. MCS: multiple cloning site.

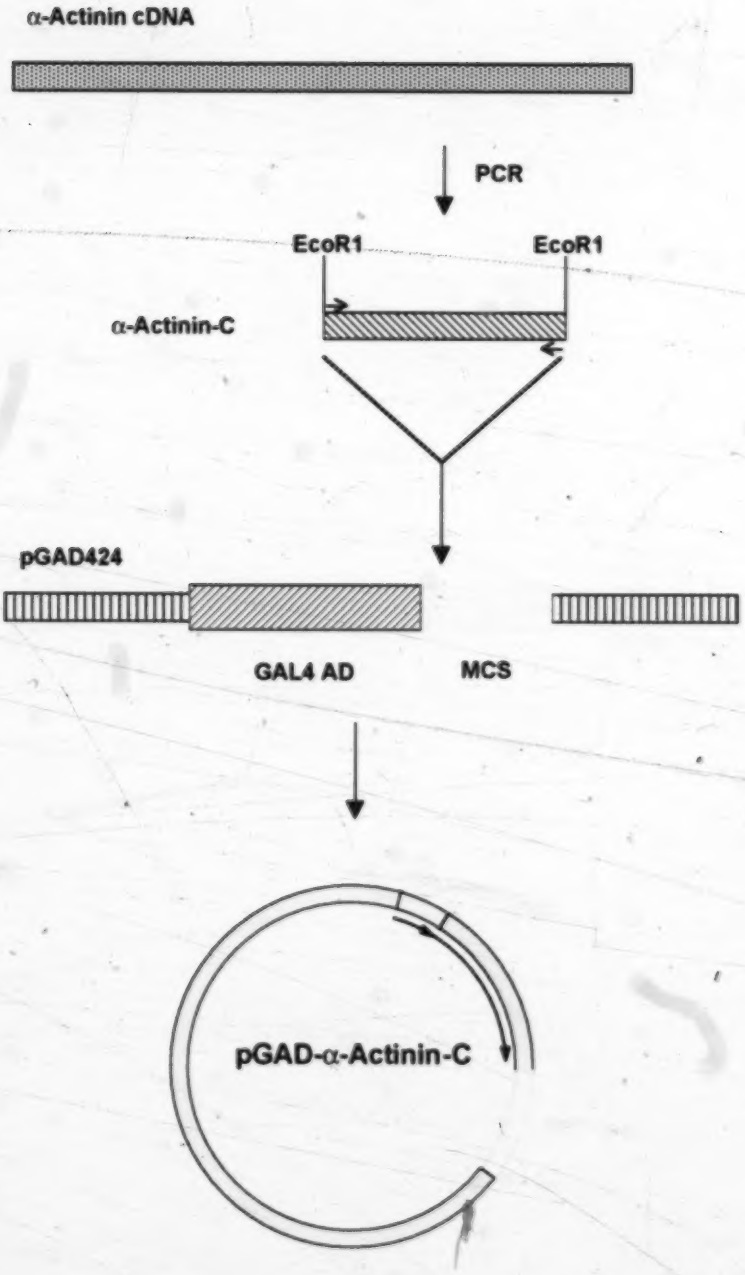
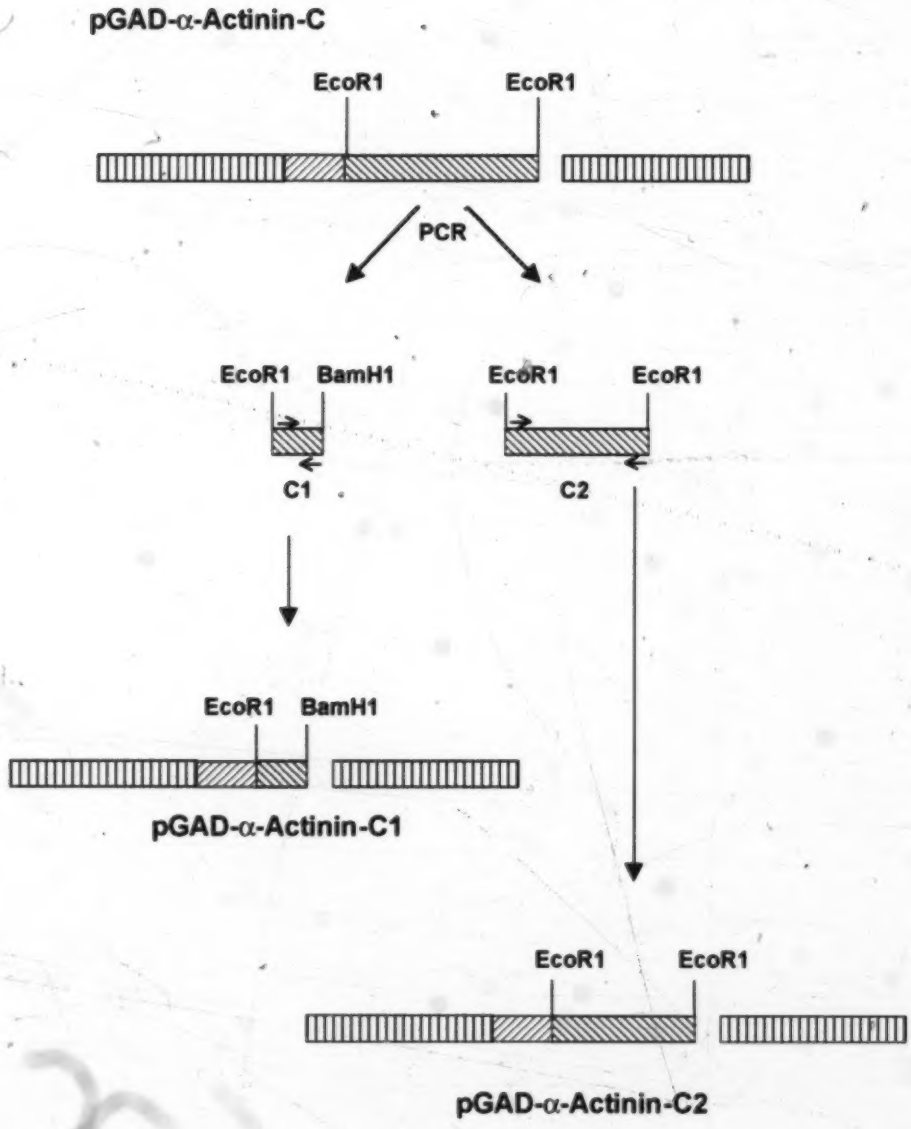

α-Actinin cDNA
PCR
EcoR1
EcoR1
α-Actinin-C
pGAD424
GAL4 AD
MCS
pGAD-α-Actinin-C

Figure 2-5. Diagrammatic Representation of the Construction of pGAD-α-Actinin-C1 and pGAD-α-Actinin-C2.

The cDNA encoding α-actinin-C1 (nucleotides 1,437-1,691) and α-actinin-C2 (nucleotides 1,692-1,776) were PCR amplified using pGAD-α-Actinin-C as template and the primers AC-3, AC-4 and AC-5, AC-6 repectively (Table 2-1) as indicated by the arrows. The PCR products C1 and C2 (shown as the blue hatched rectangles) were then inserted into the pGAD424 vector in-frame with the GAL4 DNA activation domain (green hatched rectangles), to generate the clones pGAD-α-Actinin-C1 and pGAD-α-Actinin-C2. MCS: multiple cloning site.


pGAD-α-Actinin-C
EcoR1
EcoR1
PCR
EcoR1
BamH1
EcoR1
EcoR1
C1
C2
EcoR1
BamH1
pGAD-α-Actinin-C1
EcoR1
EcoR1
pGAD-α-Actinin-C2

domain vector (pGAD10). The library was titred and found to be approximately 1.95 x 10^9 cfu/ml. The library was diluted 10,000 fold and 100 µl of diluted DNA was plated onto selective media (LB agar containing 100 µg/ml of ampicillin) and grown at 37°C for 16 hr. The resulting colonies were scraped into 2 litres of LB +amp broth and incubated with constant shaking at 37°C for 4 hours, followed by a large scale isolation of the plasmid DNA (Sambrook *et al.*, 1989).

2.4.2. Testing for Autonomous Activation of the LacZ Reporter Gene.

The cDNA clones encoding the C-terminus of α-sarcoglycan and dystrophin were tested for autonomous activation of the reporter gene. To perform this pGBT9-αSG-C or pGBT9-CT-1 were transformed into the yeast strain HF7c (containing HIS3 and LacZ reporter genes under the control of the GAL4 responsive elements) and a β-galactosidase filter lift assay carried out, if activation were to occur then the transformed yeast colonies would turn a blue colour, which did not occur for either αSG-C or CT-1 expression clones.

2.4.3 cDNA Library Screening and β-galactosidase Filter Assay.

Screening of the human skeletal muscle cDNA library was carried out as recommended by the manufacturer (Clontech Laboratories). The yeast strain HF7c was co-transformed (Ito *et al.*, 1983; Schiestl and Gietz 1989; Hill *et al.*, 1991; Gietz *et al.*, 1992) with 0.5 mg of the GAL4 DNA binding domain vector pGBT9 and 0.5 mg of the cDNA muscle library in the activation domain vector (see Appendix 1). A large scale transformation was performed yielding 2.5 x 10^6 independent clones. Yeast

transformants were plated onto selection media lacking tryptophan, leucine and histidine and grown at 30°C for eight days. Both fusion vectors are targeted to the yeast nucleus (Silver *et al.*, 1984) where a functional GAL4 activator will be reconstituted upon fusion protein interaction between the target protein and a library expressed protein. After eight days the HIS^+ colonies were replica plated onto Whatman #5 paper filters, immersed in liquid nitrogen for 30 sec, allowed to dry at room temperature and then placed onto a second filter soaked in 2 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 3.34 mg/ml of X-GAL at pH 7.0). The filters were left at room temperature for eight hours in which time positive colonies turned blue. Positive colonies were immediately re-streaked onto selective agar plates and grown at 30°C. A second β-galactosidase lift assay was performed as described above to confirm the development of the blue colour.

To quantitate the protein-protein interactions a liquid culture β-galactosidase assay using ONPG (o-nitrophenyl β-D-galactopyranoside) as substrate was performed according to the manufacturers protocol (Clontech Laboratories).

2.4.4 Elimination of False Positives.

The following procedure was used to test and eliminate any false positive protein-protein interactions. The positive yeast colonies, identified as positives by a β-galactosidase filter lift assay, were grown for 3 days at 30°C in 10 ml of selection media lacking tryptophan, leucine and histidine. The cells were spun down and the pellet was washed, by spinning and resuspending with SCE (1 M sorbitol, 100 mM sodium phosphate, pH 7.0, 60 mM EDTA) containing 10 µM β-mercaptoethanol. The washed

cells were suspended in SCE containing β-mercaptoethanol followed by the addition of 3 mg of Lyticase to lyse the cell. The lysis reaction was carried out at 30°C for an hour. Formation of spheroplasts was monitored by diluting 1 µl of the lysis reaction with 9 µl of water followed by viewing under a microscope for cell lysis. Once all the cells were lysed 400 µl of 200 mM sodium hydroxide, 1% SDS was added to the lysed cells, mixed by inversion and incubated at room temperature for 5 min. The mixture was centrifuged in an eppendorf centrifuge at 16,000 xg and the supernatant containing the plasmid DNA was precipitated with 0.3 ml of isopropanol for 5 minutes and centrifuged at 16,000 xg. The resulting pellet was washed with 70% and then 95% ethanol and dried. The dried pellet containing the plasmid DNA was resuspended in 50 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and used as a template for PCR reactions.

Each yeast colony which turned a blue colour in the β-galactosidase filter lift assay was inoculated into a solution of 25% glycerol in YPD media (20 g/L Difco peptone, 10 g/L Yeast extract, 20 g/L Agar and 2% glucose), frozen and stored at -80°C.

2.4.5 Sequencing and Identification of Positive Clones.

The plasmid DNA isolated from the yeast colonies which turned blue in the β-galactosidase filter lift assay were used as a template for identification of the DNA insert present. The 5' and 3' Matchmaker insert screening primers were used: 5'-TACCACTACAATGGATGATG-3' and 5'-GTTGAAGTGAACTTGCGGGG-3', respectively. The PCR was carried out as follows; 94°C for 30 sec, 56°C for 30 sec, 72°C for 1.5 min, for 30 cycles each, followed by a final cycle of 5 minutes at 72°C. The PCR products were digested using Alu1 restriction endonuclease in a buffer containing 6 mM

Tris-HCl, pH 7.5, 50 mM NaCl, 6 mM $MgCl_2$, and 1 mM DTT. The nucleotide sequences of the PCR products were determined by DNA sequence analysis using an automated Model 373A DNA sequencer from Applied Biosystems at the University of Alberta DNA Sequencing Laboratories. The resulting nucleotide sequences were compared in GENBANK against deposited nucleotide sequences.

2.4.6 Electroporation of Plasmid DNA.

Plasmid DNA isolated from yeast positive colonies were electroporated into the *E.coli* strain HB101. Electrocompetent cells were thawed on ice and 1 µl of isolated plasmid DNA was added to 40 µl of competent cells, mixed thoroughly and incubated on ice. The mixture was transferred to an electroporation cuvette and electroporated for 1 second at 1300 Volts, 25 mA, 25 Watts, 50 µF and a resistance of 150 ohms. The cuvette was removed from the electroporator, 500 µl of LB was added and the transformed cells were plated on M9 Minimal media (90 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 18.7 mM NH_4Cl, 1 M $MgSO_4$, 20% glucose and 1 M $CaCl_2$) and incubated at 37°C for 16 hr.

2.5 SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis.

For SDS-PAGE protein samples were solubilized in a sample buffer, containing 10% glycerol, 1% β-mercaptoethanol, 2% SDS, 65 mM Tris, pH 6.8 and 1% Bromophenol blue. SDS-PAGE was carried out using 10% or 12.5% polyacrylamide gels (Laemmli, 1970), and stained with Coomassie brilliant blue (0.5 g of R250 Coomassie brilliant blue, 25% 2-propanol and 10% acetic acid) for 1 hr. The gels were

destained overnight in a destaining solution containing 7.5% acetic acid and 10% methanol. The gels were dried using Bio-Rad Cellophane (Model 583 Gel Dryer).

Alternatively, the proteins were visualized using a silver staining procedure. After electrophoresis the gels were incubated for 1 hour in 40% methanol and 10% acetic acid followed by a 30 min incubation in 10% ethanol and 5% acetic acid. The gels were incubated for 10 min in a staining solution containing 0.85 mM $K_2CR_2O_7$ and 0.33 mM H_3PO_4. The gels were then rinsed in distilled water followed by a 30 min incubation with a silver staining solution containing 3 mM $AgNO_3$. The silver staining was developed with a mixture containing freshly prepared 186 mM Na_2CO_3 and 16.65 mM formaldehyde (37%). The reaction was stopped with 1% acetic acid.

For immunoblotting the proteins were transferred onto nitrocellulose membranes using a semi-dry transfer apparatus at 240 mA for 45 min (Towbin *et al.*, 1979). The nitrocellulose membranes were blocked with 5% milk powder in PBS (137 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.2) for 30 min. The membrane was incubated with the primary antibody diluted in 1% milk powder in PBS for 2 hours. The membrane was washed twice for 5 min with PBS containing 0.05% Tween-20 followed by a 10 min wash in PBS. The secondary hydrogen peroxidase conjugated goat anti rabbit antibody was used at a 1:10,000 dilution in 1% milk in PBS. Incubation with secondary antibody was for 30 min. The membranes were then washed twice with PBS containing 0.01% Tween-20 for 15 min and once with PBS. The antibody binding was detected by an ECL reaction (Amersham) and exposure to Kodak x-ray film.

The protein standards used were Bio-Rad low range molecular weight proteins. Pre-stained markers included; phosphorylase B 102 kDa, bovine serum albumin 78 kDa, ovalbumin 49.5 kDa, carbonic anhydrase 34.2 kDa, soybean trypsin inhibitor 28.3 kDa and lysozyme 19 kDa. Bio-Rad low range unstained markers included; phosphorylase B 97.4 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa and lysozyme 14.4 kDa.

2.6 Protein Purification.

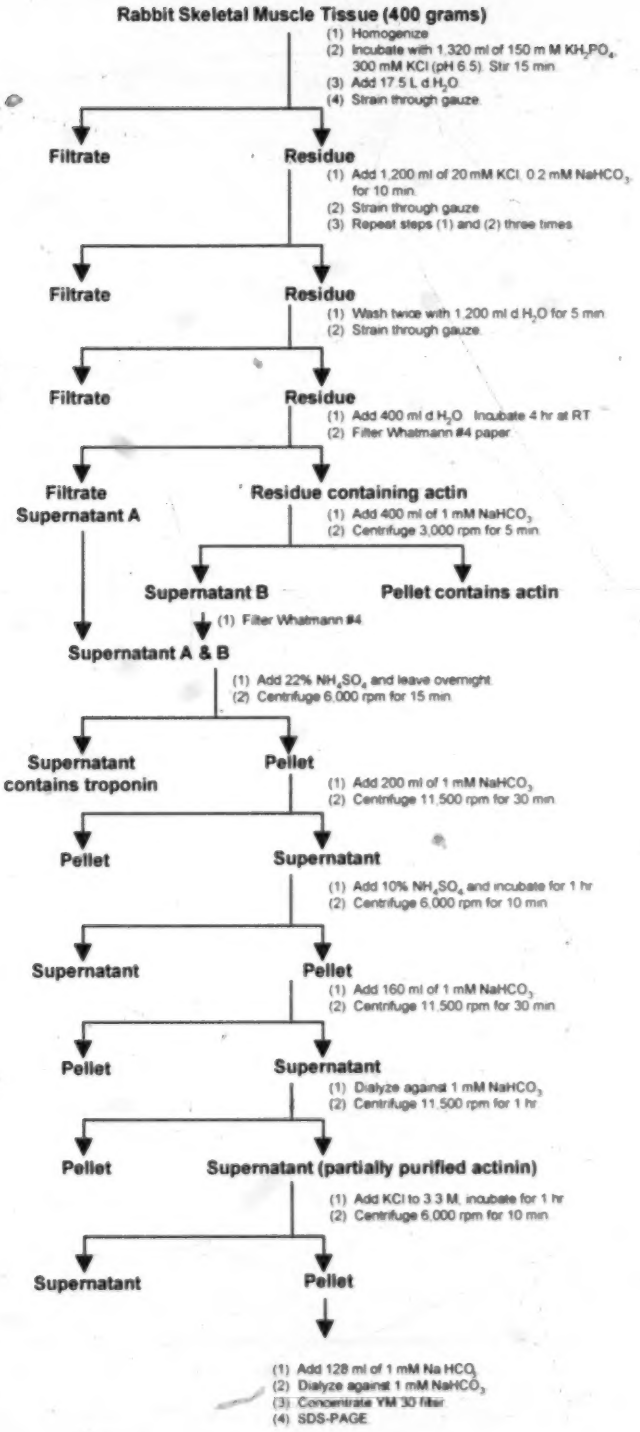
2.6.1 Purification of α-Actinin from Rabbit Skeletal Muscle.

α-Actinin was purified by a method described by Seraydarian *et al.* (1967), with some modifications. Figure 2-6 shows an outline of the procedure used. Benzamidine (0.5 M), PMSF (0.5 M), and a "cocktail" of protease inhibitors were added to all the solutions used to prevent proteolysis. The "cocktail" of protease inhibitors used were first dissolved as a 2,000 times concentrated stock solution in 100% ethanol and used at the following final concentrations: 0.01 mg/ml of aprotinin, phosphoramidone, L-1-Chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, L-Chloro-3-(4-tosylamido)-4-phenyl-2-butanone, (4-Amidinophenyl)-methanesulfonyl fluoride, N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, 0.05 mg/ml leupeptin and 0.01 mg/ml pepstatin.

Four hundred grams of back and thigh skeletal muscle were harvested from 4 month old New Zealand White rabbits. The tissue was washed with ice-cold 160 mM NaCl. The muscle was homogenized twice in 1,320 ml of 150 mM KH_2PO_4 and 300 mM KCl, pH 6.5. This mixture was left to stir slowly at 4°C for 15 min followed by the

Figure 2-6. The Purification of α-Actinin from Rabbit Skeletal Muscle.

Flow chart showing the steps involved in the purification of α-actinin from rabbit skeletal muscle. The experimental procedure is described in more detail under "Methods".


Rabbit Skeletal Muscle Tissue (400 grams)
(1) Homogenize
(2) Incubate with 1,320 ml of 150 m M KH_2PO_4, 300 mM KCl (pH 6.5). Stir 15 min
(3) Add 17.5 L d H_2O
(4) Strain through gauze
Filtrate
Residue
(1) Add 1,200 ml of 20 mM KCl, 0.2 mM $NaHCO_3$, for 10 min.
(2) Strain through gauze
(3) Repeat steps (1) and (2) three times
Filtrate
Residue
(1) Wash twice with 1,200 ml d H_2O for 5 min
(2) Strain through gauze.
Filtrate
Residue
(1) Add 400 ml d H_2O. Incubate 4 hr at RT
(2) Filter Whatmann #4 paper
Filtrate
Supernatant A
Residue containing actin
(1) Add 400 ml of 1 mM $NaHCO_3$
(2) Centrifuge 3,000 rpm for 5 min
Supernatant B
Pellet contains actin
(1) Filter Whatmann #4
Supernatant A & B
(1) Add 22% NH_4SO_4 and leave overnight
(2) Centrifuge 6,000 rpm for 15 min
Supernatant contains troponin
Pellet
(1) Add 200 ml of 1 mM $NaHCO_3$
(2) Centrifuge 11,500 rpm for 30 min
Pellet
Supernatant
(1) Add 10% NH_4SO_4 and incubate for 1 hr
(2) Centrifuge 6,000 rpm for 10 min
Supernatant
Pellet
(1) Add 160 ml of 1 mM $NaHCO_3$
(2) Centrifuge 11,500 rpm for 30 min
Pellet
Supernatant
(1) Dialyze against 1 mM $NaHCO_3$
(2) Centrifuge 11,500 rpm for 1 hr
Pellet
Supernatant (partially purified actinin)
(1) Add KCl to 3.3 M, incubate for 1 hr
(2) Centrifuge 6,000 rpm for 10 min
Supernatant
Pellet
(1) Add 128 ml of 1 mM Na HCO_3
(2) Dialyze against 1 mM $NaHCO_3$
(3) Concentrate YM 30 filter
(4) SDS-PAGE

addition of 17.5 L of H_2O. This mixture was strained through a gauze and squeezed gently. The filtrate was discarded and the residue was washed with 1,200 ml of 20 mM KCl and 0.2 mM $NaHCO_3$ for 10 min, filtered through a gauze and squeezed gently. This procedure was repeated 2 times. The residue was then washed twice with 1,200 ml of distilled H_2O for 5 min each, filtering through the gauze and gently squeezing. The residue was resuspended in 400 ml of H_2O and incubated at room temperature for 4 hr.

The following purification steps were carried out at 4°C. The residue was filtered overnight through a Whatmann #4 paper filter. The filtrate was designated Supernatant A. The residue remaining from the filtration was resuspended in 400 ml of 1 mM $NaHCO_3$ and centrifuged at 3,000 rpm for 5 min. The resulting pellet contains actin. The obtained supernatant was filtered through Whatmann #4 paper and designated Supernatant B. Supernatant A and B were combined and the proteins were precipitated by overnight incubation with 22% NH_4SO_4.

This mixture was centrifuged at 6,000 rpm for 15 min and the supernatant discarded (contains troponin). The pellet was resuspended in 200 ml of 1 mM $NaHCO_3$ and centrifuged at 11,500 rpm for 30 min. The pellet was discarded and the proteins in the supernatant were precipitated with 10% NH_4SO_4 for 1 hr. The protein mixture was centrifuged at 6,000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 160 ml of 1 mM $NaHCO_3$ and centrifuged again at 11,500 rpm for 30 min. The pellet was discarded and the supernatant was dialyzed against 1 mM $NaHCO_3$ overnight. The dialyzed sample was centrifuged at 11,500 rpm for 1 hr and the pellet discarded. The supernatant contains partially purified α-actinin. Solid KCl was added to the supernatant to a final concentration of 3.3 M followed by mixing for 1 hr. The

Figure 2-7. Analysis of the Purified Rabbit Skeletal Muscle α-Actinin.

α-Actinin was purified from the skeletal muscle of New Zealand white rabbits as outlined under "Methods". Proteins were separated by SDS-PAGE (10%) and visualized by Coomassie blue staining. Lane 1, protein sample before the final NH_4SO_4 precipitation (see Figure 2-6); lane 2, purified α-actinin; lane 3, concentrated purified α-actinin. α-Actinin and actin are indicated by arrows. The position of the Bio-Rad molecular weight markers are indicated.

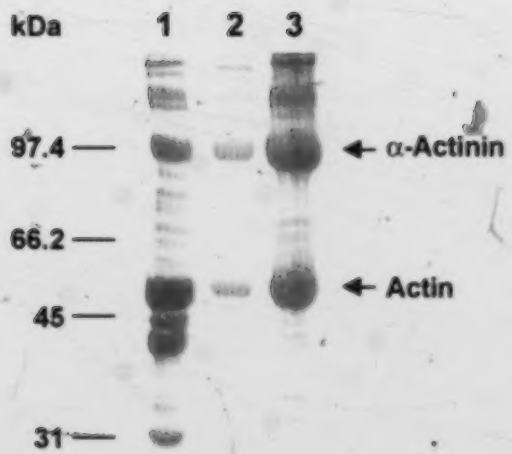
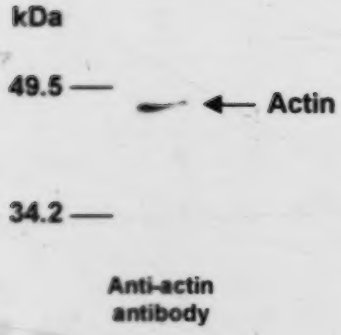

kDa
1
2
3
97.4
66.2
45
31
α-Actinin
Actin

Figure 2-8. Purified Rabbit Skeletal Muscle α-Actinin Contains Actin.

α-Actinin was purified from the skeletal muscle of New Zealand white rabbits as outlined under "Methods". Proteins were separated by SDS-PAGE (10%), transferred to nitrocellulose and tested for reactivity with an anti-actin antibody. Actin is indicated by the arrow. The position of the Bio-Rad molecular weight markers are indicated.


kDa
49.5
Actin
34.2
Anti-actin
antibody

mixture was centrifuged at 6,000 rpm for 10 min and the supernatant discarded. The pellet was dissolved in 128 ml of 1 mM $NaHCO_3$ followed by dialysis against 1 mM $NaHCO_3$ for 16 hr.

The dialyzed proteins were concentrated using an Amicon concentrator with a YM30 filter (molecular weight cut-off 30,000) followed by quantitation of the protein. The purity of the α-actinin protein was estimated by SDS-PAGE, followed by Western immunoblot analysis. Figure 2-7 shows a typical SDS-PAGE of the purified α-actinin. A 45 kDa protein band is always present in the α-actinin preparation. Western analysis with an anti-actin antibody revealed its identity was actin (Figure 2-8).

2.6.2 Expression and Purification of the C-terminus of α-Sarcoglycan Fusion Protein.

The last 75 amino acids at the carboxyl end of α-sarcoglycan (Figure 2-1) were expressed as a glutathione-S-transferase (GST) fusion protein. PCR was used to synthesize the cDNA encoding the carboxyl 75 amino acids (amino acid residues 312-387) of α-sarcoglycan to generate GST-αSG-C (Figure 2-9). The template used was the pGBT9-αSG-C clone generated as described previously. SG-3 and SG-4 (Table 2-1) were used as primers. The PCR cycle parameters were 94°C for 1 min, 64°C for 1 min, 72°C for 2 min, for a total of 25 cycles, followed by one cycle at 72°C for 10 min. The PCR product (249 base pairs in length) was cloned into the GST expression vector pGEX-3X at BamH1 and EcoR1 endonuclease restriction sites. The pGEX-3X-GST-αSG-C sequence was confirmed by DNA sequencing.

The pGEX-3X-GST-αSG-C vector was transformed into DH5α for protein expression. The transformed cells were grown in 200 ml of LB containing 100 μg/ml ampicillin for 16 hr at 37°C, transferred to 1 litre of LB+amp, and grown to mid-log phase (A_{600}=0.6-1.0). Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After four hours the cells were centrifuged at 3,500 rpm for 15 min at 4°C. The pellet was resuspended in 40 ml PBS, 0.1% Triton X-100, 0.5 mM PMSF, 0.5 mM Benzamidine and protease inhibitor cocktail. The cells were lysed using a French press set at 1,000 psi followed by centrifugation for 10 min at 10,000 rpm at 4°C. The supernatant and pellet were analyzed for α-SG-C protein expression with anti-C-terminal α-sarcoglycan antibodies. Figure 2-10 B shows that the α-sarcoglycan antibody recognizes a 35 kDa protein which corresponds to the predicted molecular weight of the GST-αSG-C fusion protein. Figure 2-10 B shows a small amount of insoluble GST-αSG-C left in the pellet.

The GST-αSG-C fusion protein was purified by glutathione sepharose 4B affinity chromatography. The *E.coli* supernatant was diluted with PBS containing 0.1% Triton X-100, to a final protein concentration of 2 mg/ml and applied to the column equilibrated in the same buffer. The GST fusion proteins were eluted with 5 mM glutathione and 50 mM Tris-HCl, pH 8.0 and the eluante was collected on ice. The eluted protein was concentrated in an Amicon concentrator with an Amicon Diaflo Ultrafilter YM10 (molecular weight cut-off 10,000). Figure 2-11 shows the GST-αSG-C fusion protein detected by Western blot analyses with an anti-α-SG-C antibody.

The GST-αSG-C fusion protein was also expressed in BNN103, a protease deficient *E.coli* strain, and a Topp2 strain. The transformed cells were grown at 30°C in a

nutrient rich media, YTA (Tryptone 16 g/L, Yeast extract 10 g/L and NaCl 5 g/L, pH 7.0). Purification of the fusion protein was carried out as described above. Figure 2-12 shows a SDS-PAGE analysis of the purified protein. All the purified proteins were aliquoted and stored at -80°C until further use.

2.6.3 Cloning, Expression and Purification of the C-Terminus of Dystrophin as a GST Fusion Protein.

The last 201 amino acids at the C-terminus of dystrophin were expressed as a GST fusion protein and named GST-CT-1. The cloning, expression and purification of GST-CT-1 is described by Milner *et al.* (1992). Briefly, the cDNA encoding the carboxyl 201 amino acids of dystrophin were subcloned into a pGEX-3X plasmid in-frame with GST. The pGEX-3X plasmids were transformed into the *E.coli* strain BNN103. Expression of the GST fusion protein was induced with IPTG followed by Glutathione-Sepharose 4B affinity column chromatography. The eluted fusion proteins were dialysed against 50 mM Tris, pH 7.1 containing 1 mM EDTA and subjected to further purification by MonoQ FPLC chromatography. The identity of the fusion proteins was confirmed by Western blot analysis using anti-dystrophin antibodies (Figure 2-13). It has been documented previously in our laboratory that the lower protein band is a truncated version of GST-CT-1 (Milner *et al.*, 1992).

2.6.4 Determination of Protein Concentration.

Protein concentration was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard in these assays.

Figure 2-9. Diagrammatic Representation of the Construction of the GST Fusion Protein α-Sarcoglycan-C.

The cDNA encoding GST-αSG-C was PCR amplified using pGBT9-αSG-C as template and the primers SG-3 and SG-4 (Table 2-1) as indicated by the arrows. The PCR product (blue hatched rectangle) was then inserted into the pGEX3X vector in-frame with the GST encoded protein (green hatched rectangle) at BamH1 and EcoR1 restriction endonuclease sites. The plasmid was designated pGEX-3X-GST-αSG-C. The arrows shown in the circular plasmid indicate the translated protein sequences. GST: glutathione S-transferase. MCS: multiple cloning site.

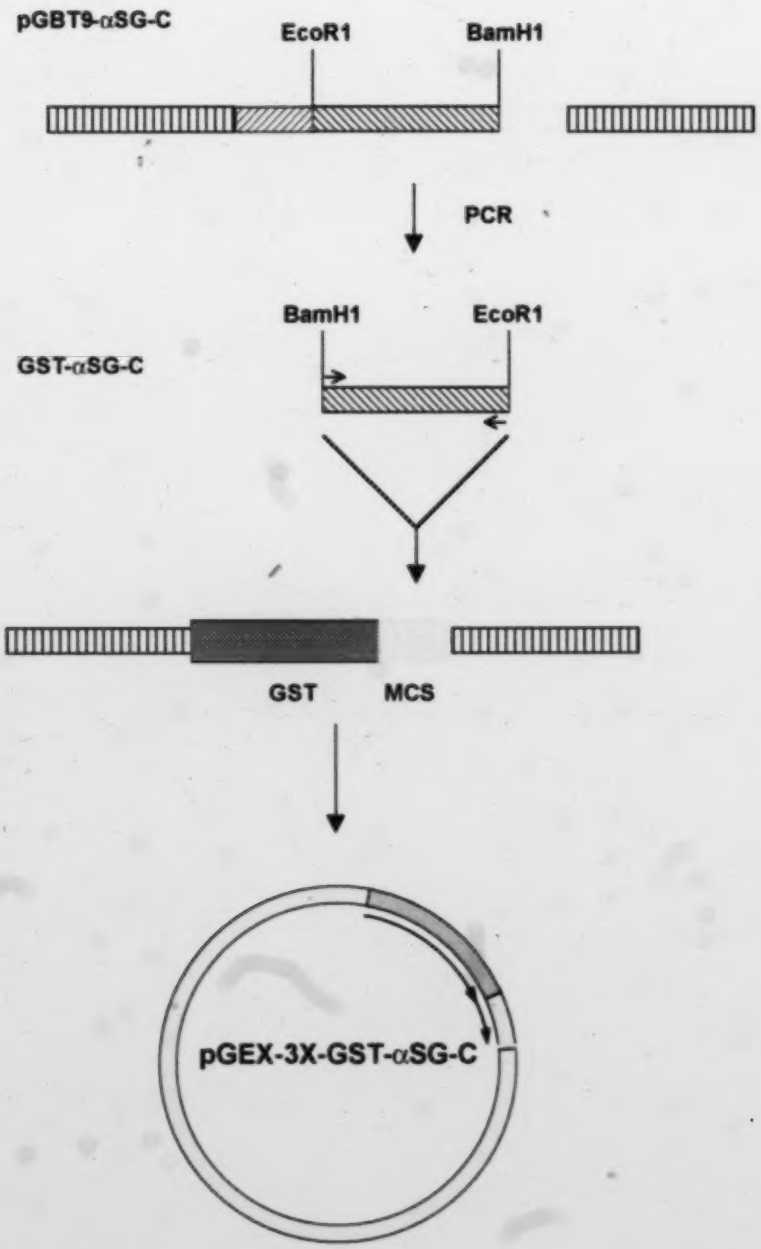
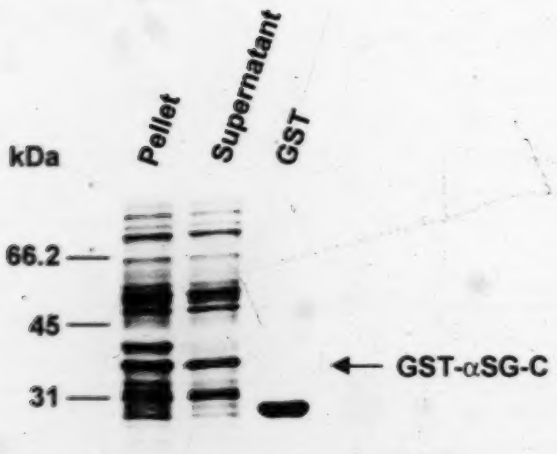

pGBT9-αSG-C
EcoR1
BamH1
PCR
BamH1
EcoR1
GST-αSG-C
GST
MCS
pGEX-3X-GST-αSG-C

Figure 2-10. Identification of the C-terminus of α-Sarcoglycan GST Fusion Protein. The last 75 amino acids of α-sarcoglycan were expressed as a GST fusion protein. The transformed DH5α cells were induced with IPTG to express the proteins, then French pressed and centrifuged. The supernatant and pellet were separated on a 10% SDS-PAGE. **A**, Coomassie blue stain of the SDS-PAGE. **B**, Western immunoblot incubated with a 1:100 dilution of anti-α-sarcoglycan antibody. The 35 kDa GST-αSG-C fusion protein is indicated by the arrow. The position of the Bio-Rad molecular weight markers are indicated.

A


kDa
Pellet
Supernatant
GST
66.2
45
31
GST-αSG-C

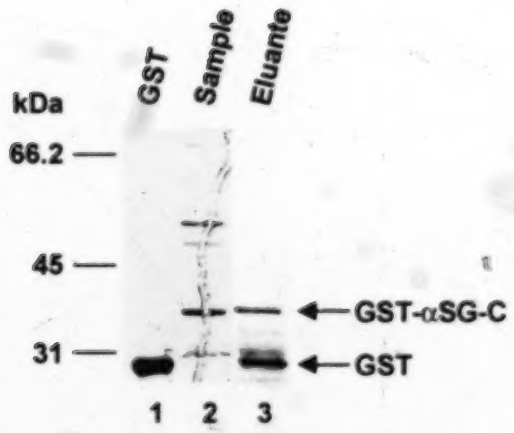
B

Pellet
Supernatant
kDa
34.2
28.3
GST-αSG-C

Figure 2-11. SDS-PAGE Analysis of α-Sarcoglycan-C GST Fusion Protein.

The GST-αSG-C fusion protein was expressed in DH5α *E.Coli* cells, and purified by Glutathione-Sepharose column chromatography. The eluted proteins were separated on a 10% SDS-PAGE followed by Coomassie blue staining and Western blot analysis with anti-α-sarcoglycan antibodies. **A**, Coomassie stained gel. Lane 1, recombinant GST; lane 2, sample applied on the Glutathione-Sepharose column; lane 3, eluted GST fusion proteins. **B**, Western blot analysis of the same sample. Lane 1, recombinant GST; lane 2, sample applied on the Glutathione-Sepharose column; lane 3, eluted GST fusion proteins. The arrow indicates the position of GST-αSG-C and GST. The position of the Bio-Rad molecular weight markers are indicated.

A


kDa
GST
Sample
Eluante
66.2
45
31
GST-αSG-C
GST
1
2
3

B

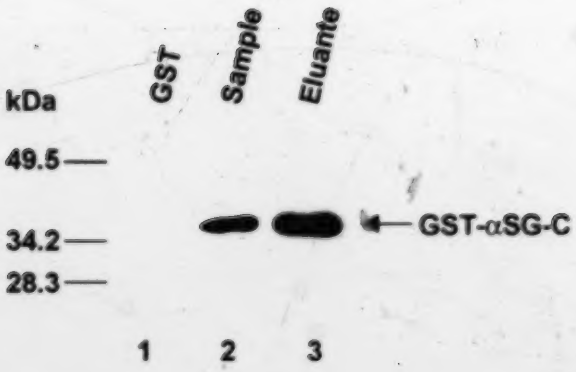
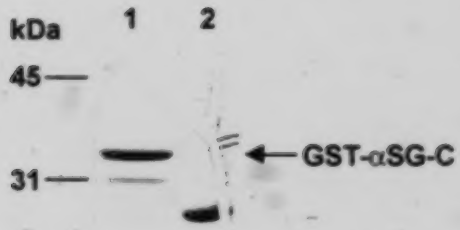

kDa
GST
Sample
Eluante
49.5
34.2
28.3
GST-αSG-C
1
2
3

Figure 2-12. The Expression and Purification of GST-αSG-C in BNN103 and Topp2 strains of *E.coli*.

pGEX3X-GST-αSG-C was transformed into either BNN103 or Topp2 strains of *E.coli*. Protein expression was induced with IPTG, followed by Glutathione-Sepharose column chromatography. The concentrated proteins were separated on a 12.5% SDS-PAGE followed by Coomassie blue staining. **A**, BNN103 *E.coli* expressed fusion proteins. Lane 1, concentrated GST fusion protein from the Glutathione-Sepharose column. Lane 2, recombinant GST. **B**, Topp2 *E.coli* cells transformed with GST-αSG-C. Lane 1, concentrated GST fusion protein from the Glutathione-Sepharose column. Lane 2, recombinant GST protein. The location of GST-αSG-C is indicated by the arrow. The position of the Bio-Rad molecular weight markers are indicated.

A



B

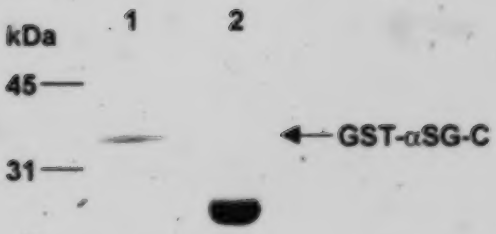
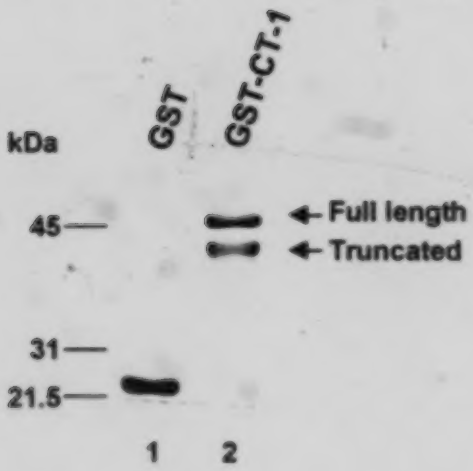


Figure 2-13. Protein Expression of GST-CT-1.

GST-CT-1 fusion protein was expressed and purified as described under "Methods". Coomassie blue stain of a 10% SDS-PAGE. Lane 1, recombinant GST; lane 2, purified GST-CT-1. Full length and truncated GST-CT-1 protein are indicated by arrows. The position of the Bio-Rad molecular weight markers are indicated.


kDa
GST
GST-CT-1
45
31
21.5
Full length
Truncated
1
2

2.7 Isolation of Sarcolemma Membrane Vesicles.

Heavy microsomes were isolated from rabbit skeletal muscle as described by Ohlendieck *et al.* (1991c). To purify the sarcolemma vesicles, the heavy microsomes were diluted to 1 mg/ml of protein and agglutinated with 1 mg/ml of wheat germ lectin in a buffer containing 160 mM NaCl and 10 mM sodium phosphate, pH 6.8 (Charuk *et al.*, 1989). The mixture was incubated on ice for 10 min and centrifuged for 1 min at 16,000 xg in an eppendorf centrifuge. The pellet contained lectin-agglutinated sarcolemma vesicles. The pellet was washed 3 times with 250 mM sucrose and 10 mM histidine, pH 7.5. The wheat germ agglutinated sarcolemma vesicles were de-agglutinated with 200 mM N-acetyl-D-glucosamine in the same buffer (Charuk *et al.*, 1989; Ohlendieck *et al.*, 1991c). Membrane vesicles were pelleted by centrifugation for 60 min at 120,000 xg. The pellet containing purified sarcolemma proteins were resuspended in 50 mM sodium phosphate and 160 mM NaCl, pH 7.5. The wheat germ treated sarcolemma vesicles were frozen and stored at -80°C.

2.8 Antibody Production.

Polyclonal antibodies against a synthetic peptide corresponding to the C-terminus of α-sarcoglycan were raised in rabbits. A synthetic peptide corresponding to amino acid residues 376-387 (NH_2-D-S-A-Q-V-P-L-I-L-D-Q-H-COOH) at the C-terminus of α-sarcoglycan (Figure 2-1) was synthesized at the Alberta Peptide Institute as a keyhole limpet haemocyanin (KLH) and BSA conjugate.

One mg/ml of KLH conjugated synthetic peptide was emulsified in 2 ml of Freund's complete adjuvant and injected subcutaneously into two New Zealand white

rabbits. After two weeks the immunization was repeated with 1 mg/ml of synthetic peptide emulsified with 2 ml of Freund's incomplete adjuvant. The sera was tested for immunoreactivity against the BSA conjugated synthetic peptide by Western blot analysis. The blood was collected by cardiac puncture. The serum was collected and stored in aliquots at -80°C.

Antibodies against α-sarcoglycan were affinity purified on a BSA-conjugated synthetic peptide Sepharose column chromatography. One mg/ml of BSA conjugated synthetic peptide was dissolved in coupling buffer (100 mM $NaHCO_3$, pH 8.3 and 500 mM NaCl). The CNBr-activated Sepharose 4B was equilibrated with 1 mM HCl. A ratio of 5 ml of coupling buffer containing BSA conjugated peptide was added to 1 gram of CNBr-activated Sepharose beads, and left rotating end over end for 2 hr at room temperature. Excess, unbound ligand was washed by successive spins in coupling buffer. The remaining active groups were blocked with 100 mM Tris-HCl, pH 8.0 for 2 hr at room temperature. The beads were then washed with 100 mM acetate buffer, pH 4.0 containing 500 mM NaCl, then with 100 mM Tris buffer, pH 8.0 containing 500 mM NaCl. For antibody purification synthetic peptide coupled beads were washed with 2-4 bed volumes of elution buffer (Immunopure Ag/Ab Elution buffer) followed by a wash with 5 bed volumes of TBS (100 mM Tris-Cl, pH 8.0 and 150 mM NaCl). The serum containing the anti-α-sarcoglycan antibodies was diluted 1:3 in TBS, added to the beads and left rotating overnight at 4°C. The beads were washed with 2 bed volumes of TBS containing 500 mM NaCl, followed by a wash with 2 bed volumes of TBS. The antibody was eluted with 5 bed volumes of Immunopure Elution buffer directly into a tube containing a solution of 1 mg/ml of BSA to stabilize the antibody. The affinity purified

antibody was concentrated by centrifugation at 6,000 rpm for 30-60 min in a Millipore "Ultrafree" tube (molecular weight cut-off 30,000).

Anti-dystrophin C-terminus antibodies were made in rabbits against a synthetic peptide, NH_2-P-S-S-R-G-N-T-P-G-K-P-COOH, corresponding to amino acid residues 3,668-3,679 of the C-terminus of dystrophin. The peptide was synthesized at the Alberta Peptide Institute and coupled to KLH and BSA, as described by Milner *et al.* (1992).

Rabbit polyclonal antibodies to the α-actinin-2 isoform were prepared as described by Chan *et al.* (1998). Rabbits were injected with the synthetic peptide, NH_2-N-Q-I-E-P-G-V-Q-Y-N-Y-V-Y-D-E-D-E-COOH, corresponding to amino acids 2-18 of the N-terminus of human α-actinin-2. This antibody was a generous gift from Dr. A. H. Beggs, Harvard Medical School, Boston.

2.9 Testing the Sensitivity of the α-Sarcoglycan Antibody.

The affinity purified antibody to the C-terminus of α-sarcoglycan (1:50 dilution) was tested against the BSA conjugated synthetic peptide and against purified sarcolemma vesicles. Fourty µg of sarcolemma vesicles and 10 µg of BSA-peptide were loaded onto a 10% SDS-PAGE, the separated proteins were then transferred to nitrocellulose and probed with a 1:50 dilution of affinity purified antibodies. Figure 2-14 shows that the affinity purified α-sarcoglycan antibody recognizes a 50 kDa protein corresponding to the size of full length α-sarcoglycan.

2.10 Protein Overlay Assay.

For the protein overlay assay purified α-actinin (50 μg) was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose membrane was cut into 2 cm strips for the overlay assay. The strips were blocked overnight by incubation in a binding buffer containing 20 mM Hepes, pH 7.0, 2 mM $MgCl_2$, 150 mM KCl, 0.1 mM EGTA, 0.1 mM $CaCl_2$ and 5% BSA. Thirty μg of GST-CT-1 was added to freshly made blocking buffer and incubated with the nitrocellulose blots for 2 hours at room temperature. Blots were washed with the binding buffer 3 times for 5 min, followed by two 30 min washes with binding buffer. The blots were then incubated with an anti-C-terminal dystrophin antibody at a dilution of 1:100 in the binding buffer for 2 hr. The blots were then washed two times for 15 min in a binding buffer (without BSA) containing 0.1% Tween, followed by a 15 min wash with the binding buffer alone. A secondary goat anti-rabbit peroxidase antibody was added to the blots at a 1:10,000 dilution in the binding buffer. The reaction was developed with ECL reagents and visualized by exposure to Kodak X-ray film.

2.11 Isolation of Plasma Membranes.

C_2C_{12} muscle cells were grown in DMEM containing 10% FBS at 37°C with 5% CO_2. The cells were grown in 10 cm culture dishes coated with 0.1% gelatin. Differentiation of myoblasts (at 80% confluency) into myotubes was induced by replacing 10% FBS with 2% horse serum. After 7 days in culture the plasma membranes were isolated as outlined in Figure 2-15 using positively charged silica beads. Once the cells were ruptured they were then passed through a Nycodenz gradient which separates

Figure 2-14. Immunoreactivity of Skeletal Muscle Sarcolemma Proteins with Anti-α-Sarcoglycan Antibody.

A rabbit polyclonal antibody was made against a synthetic peptide (NH_2-D-S-A-Q-V-P-L-I-L-D-Q-H-COOH) corresponding to the C-terminus of α-sarcoglycan. The affinity purified antibody, anti-α-sarcoglycan was used for immunoreactivity. Lane 1, BSA-conjugated synthetic peptide (NH_2-D-S-A-Q-V-P-L-I-L-D-Q-H-COOH). Lane 2, sarcolemma vesicles purified from rabbit skeletal muscle. The arrow depicts a 50 kDa protein band corresponding to the molecular size of the α-sarcoglycan protein. The lower molecular weight protein band may be a degradation product of α-sarcoglycan. The position of the Bio-Rad molecular weight markers are indicated.

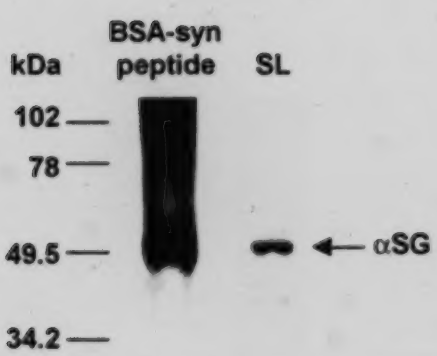
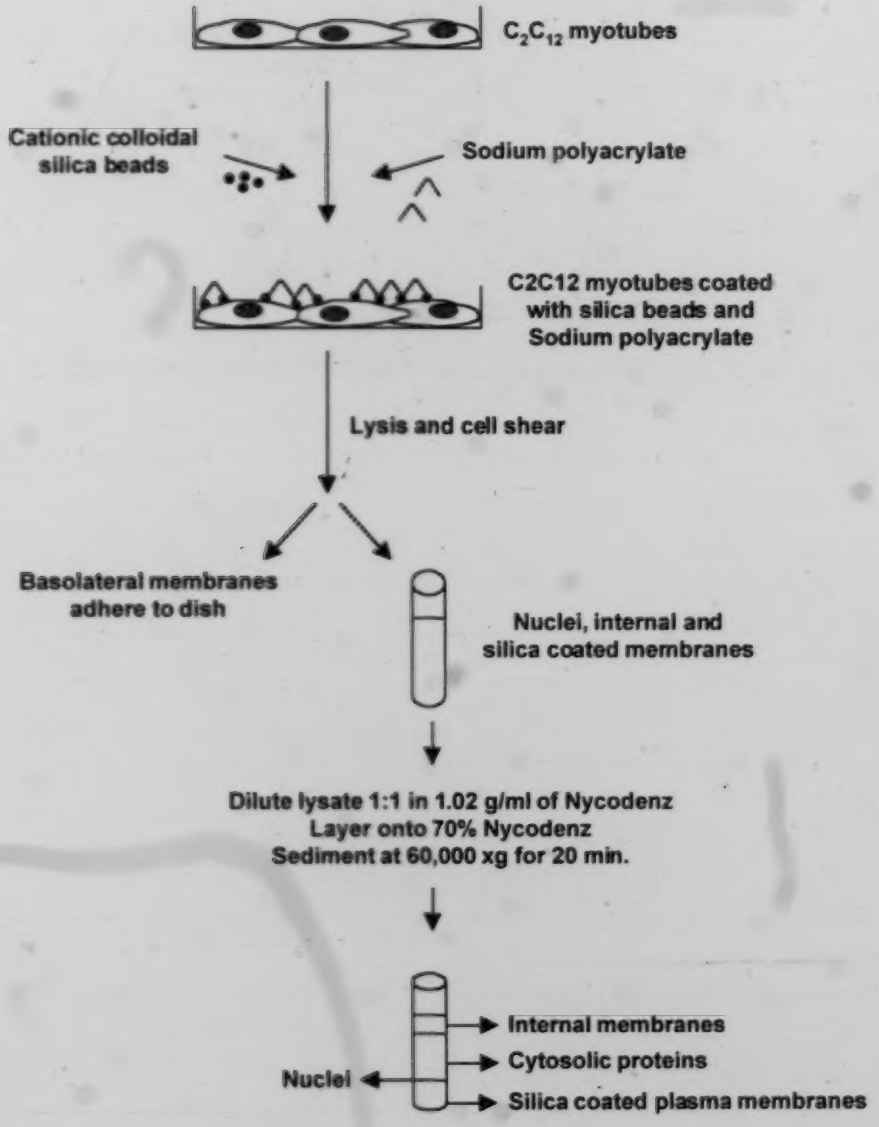

BSA-syn
peptide
kDa
SL
102
78
49.5
34.2
αSG

Figure 2-15 Flow Diagram Showing the Steps Involved in the Isolation of Plasma Membranes from C_2C_{12} Myotubes.


C_2C_{12} myotubes
Cationic colloidal silica beads
Sodium polyacrylate
C2C12 myotubes coated with silica beads and Sodium polyacrylate
Lysis and cell shear
Basolateral membranes adhere to dish
Nuclei, internal and silica coated membranes
Dilute lysate 1:1 in 1.02 g/ml of Nycodenz
Layer onto 70% Nycodenz
Sediment at 60,000 xg for 20 min.
Internal membranes
Cytosolic proteins
Nuclei
Silica coated plasma membranes

out the various cellular fractions, leaving purified plasma membranes attached to the silica beads (Stolz and Jacobson, 1992). Prior to SDS-PAGE, proteins were acetone precipitated in 3 volumes of acetone at -20°C. All tissue culture procedures were carried out in sterile hoods and all tissue culture solutions were sterilized by either filtering or autoclaving.

2.12 Immunofluorescence Microscopy.

Human trapesius muscle was flash frozen in liquid nitrogen cooled isopentane and cut into 10 micron section on a Micron 505 cryostat. For double-label analysis, sheep anti-30 kDa dystrophin and rabbit α-actinin-2 antibodies (both 1 μg/ml) were visualized using FITC-labeled donkey anti-sheep IgG (1:200) and Cy3 goat anti-rabbit IgG at 1:3,000. Stained sections were analyzed using a Leica TCS-NT confocal microscope using a 100x objective and AOTF filter to selectively visualize each label without bleed through. The FITC label was excited at 488 nm and Cy3 at 589 nm. Images were collected in serial planes throughout the depth of the section at 1024 x 1024 pixel resolution. Images stacks were imported into Image Space (Molecular Dynamics, Sunnyvale, CA) for subsequent rendering. Lookthrough projections were generated for each protein and superposed for qualitative analysis. This technique was performed by Simon Watkins at the University of Pittsburg, Pennsylvania.

CHAPTER THREE

IDENTIFICATION OF PROTEINS INTERACTING WITH THE C-TERMINAL CYTOPLASMIC REGION OF α-SARCOGLYCAN

3.1 Introduction.

α-Sarcoglycan is a member of the sarcoglycan family of proteins which also includes β-, γ-, and δ-sarcoglycan (Henry and Campbell, 1996). The sarcoglycans are components of the DGC and are known to be vital for maintaining the stability and function of the DGC (Worton, 1995). The lack of expression of α-sarcoglycan at the sarcolemma results in SCARMD and the remaining sarcoglycans are underexpressed (Ben Hamida *et al.*, 1983; Ben Jelloun-Dellagi *et al.*, 1990). α-Sarcoglycan is an important protein in maintaining muscle stability and integrity (Worton, 1995). To understand the function of α-sarcoglycan, we investigated the role the C-terminus plays in muscle function. We employed the yeast two-hybrid system to identify protein-protein interactions with the C-terminus of α-sarcoglycan. To date there have been no reports of proteins interacting with this C-terminus and the function for this protein has not been determined.

The Matchmaker two-hybrid system is based on the two-hybrid assay which was first developed by Stanley Fields *et al.* (Fields and Song, 1989). The two-hybrid assay is used to detect genes encoding proteins that interact with a protein of interest. There have been many discoveries of specific protein-protein interactions by screening mammalian and yeast cDNA libraries (Durfee *et al.*, 1993; Vojtek *et al.*, 1993; Guan *et al.*, 1994; Miyata *et al.*, 1994; Rothe *et al.*, 1994; Song *et al.*, 1994). The screening procedure involves reconstitution of a functional GAL4 transcriptional activator and consequent activation of a reporter gene, HIS3, which is under the control of a GAL4 responsive promoter.

3.2 Results.

3.2.1 Identification of Proteins Interacting with the C-terminus of α-Sarcoglycan.

The Matchmaker yeast two-hybrid system was employed to identify potential proteins binding to the C-terminus of α-sarcoglycan (amino acid residues 312-387, see Figure 2-1). A human skeletal muscle cDNA library was screened with the pGBT9 vector containing cDNA encoding α-SG-C. Positive yeast colonies were identified using a β-galactosidase filter lift assay (Figure 3-1). An eight hour maximum incubation time was set as a limit for identification of positive colonies. Using this approach 400 positive colonies were identified. A second lift assay was carried out and a total of 13 clones were eliminated as false positives, since they failed to utilize β-galactosidase as a substrate. An additional 15 clones were eliminated because of their lack of growth in the selection media. The remaining 372 positive clones were analyzed by PCR as described under "Methods" and the PCR products were subjected to restriction endonuclease digestion of Alu1, in order to identify related or identical clones. A number of PCR products were analyzed for their nucleotide sequence and further analyzed by BLAST searches to determine the identity of the clones.

Figure 3-2 A shows restriction endonuclease digestion patterns of seven positive clones. Clone # 60 has been identified as cDNA encoding a fragment of a Na^+/K^+ ATPase, the remaining clones all show variation in their digestion patterns. Figure 3-2 B shows the Alu1 restriction endonuclease digestion pattern of three identical clones. Clone number 234, 342 and 357 have been sequenced and match to the Cold Shock Domain region in the dbpA protein (Kudo *et al.*, 1995).

Figure 3-1. β-Galactosidase Filter Lift Assay of the C-terminus of α-Sarcoglycan and the Cold Shock Domain Protein, DbpA.

The human skeletal muscle cDNA library was screened with the cDNA encoding the C-terminus of α-sarcoglycan. **A**, yeast cells transformed with the positive control vector pCL1; **B**, yeast co-transformed with pGBT9 and pGAD control vectors; **C**, yeast transformed with pGBT9-αSG-C (the last 75 amino acids of the human α-sarcoglycan protein); **D**, yeast co-transformed with pGBT9-αSG-C and pGAD; **E**, yeast positive colonies containing pGBT9-αSG-C and the positive clone #342 (nucleotides 319-432 in the dbpA protein which corresponds to the Cold Shock Domain); **F**, yeast positive colonies containing pGBT9-αSG-C and the positive clone #390 (nucleotides 319-531 in the dbpA protein).

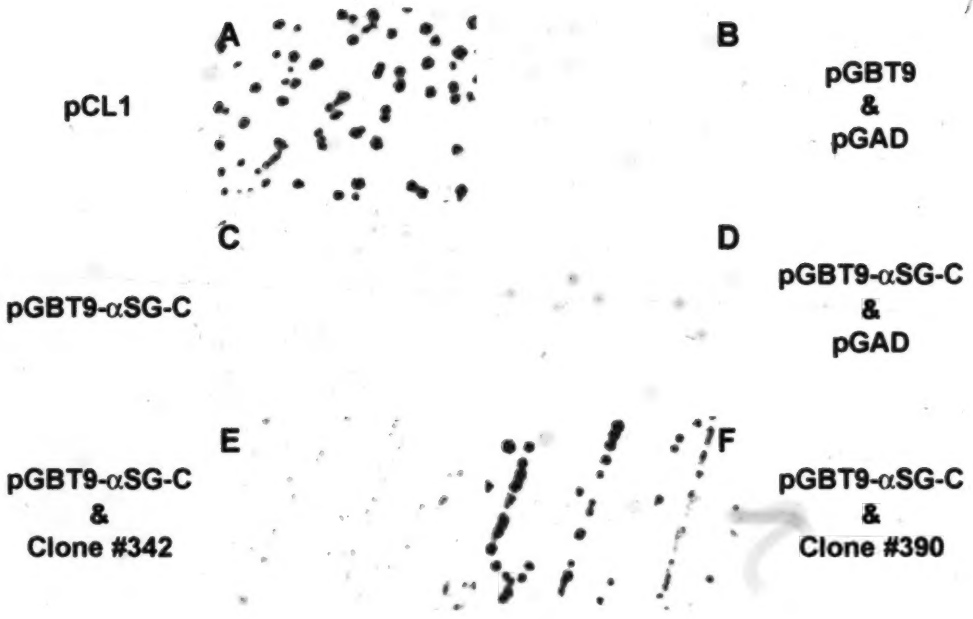
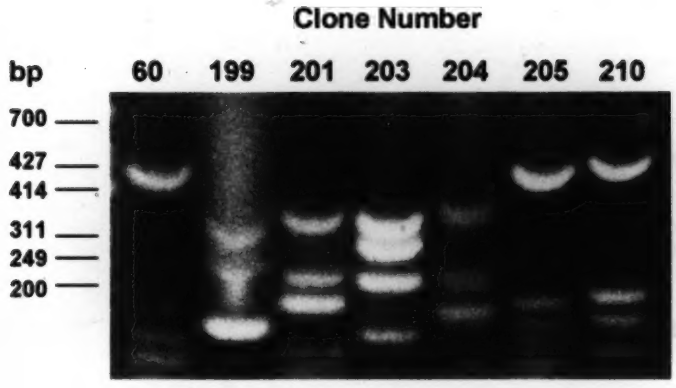

A
pCL1
B
pGBT9
&
pGAD
C
pGBT9-αSG-C
D
pGBT9-αSG-C
&
pGAD
E
pGBT9-αSG-C
&
Clone #342
F
pGBT9-αSG-C
&
Clone #390

Figure 3-2. Restriction Enzyme Digestion of the PCR Products from Positive Clones Isolated from a Yeast Two-Hybrid Human Skeletal Muscle Library Screening with α-Sarcoglycan-C.

The yeast two-hybrid system was used to screen the human skeletal muscle cDNA library with the plasmid containing the cDNA encoding the C-terminus of α-sarcoglycan. DNA from selected positive clones were isolated and amplified by PCR. The PCR products were subjected to AluI restriction endonuclease digestion. The DNA fragments were separated on a 2% agarose gel and visualized using ethidium bromide. **A**, endonuclease digestion pattern of clone number 60 (Na^+/K^+ ATPase), 199, 201, 203, 204, 205, and 210. **B**, endonuclease digestion pattern of clone number 234, 342, and 357, all of which correspond to Cold shock domain clones. Base pairs (bp) are indicated.

A


Clone Number
bp
60
199
201
203
204
205
210
700
427
414
311
249
200

B

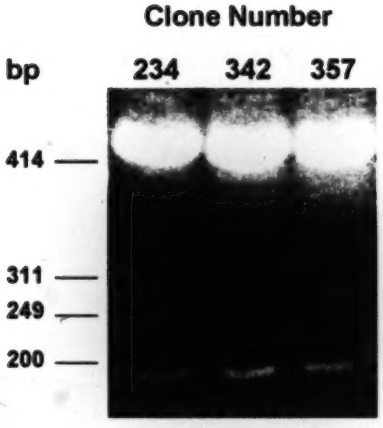

Clone Number
bp
234
342
357
414
311
249
200

Table 3-1 shows that a total of 36 positive clones have been analyzed for their nucleotide sequence and identified. Five clones matched to ribosomal or mitochondrial proteins. Ribosomal proteins have the ability to interact with hydrophobic or charged residues and are commonly reported false positives found from library screens (see WEB site reference). Fourteen clones that were analyzed for their nucleotide sequence did not contain any insert. Tropomyosin and human skeletal muscle α-actin were not in-frame with the GAL4 DNA activation domain, and therefore were discarded as false positives. A total of nine clones did not match to deposited nucleotide sequences in the Genbank, and may therefore, encode candidate novel proteins that bind to the C-terminus of α-sarcoglycan. Identification of these proteins needs to be carried out in more detail.

One clone matched to a short region of the Na^+/K^+ ATPase which was in-frame with the GAL4-DNA binding domain. Analysis of this clone (clone #60) showed it encoded the amino acid residues 117-182 of the α2-subunit of the Na^+/K^+ ATPase. It was a surprising finding because the α-2-subunit of the Na^+/K^+ ATPase is an integral membrane protein (Shull *et al.*, 1989).

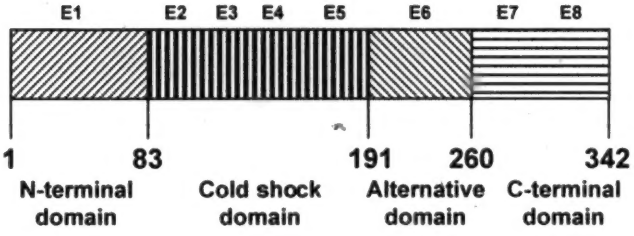
Figure 3-2 B shows three clones with a distinctive two band pattern, these clones were sequenced and found to all match to the Cold Shock Domain (CSD) found in the DNA binding protein, dbpA (Figure 3-3) (Kudo *et al.*, 1995). The dbpA contains a highly conserved CSD encoded by exon 2-5 (amino acid number 106-155) (Figure 3-3). The four clones identified as dbpA interacting with α-SG (clone #234, 342, 357, 390), encode amino acid number 107-177 of this CSD. Analysis of the AluI restriction endonuclease digestion patterns of the remaining positive clones showed at least 68 clones with the identical digestion pattern (Kudo *et al.*, 1995).

Table 3-1. A List of Selected Positive Clones Isolated from the Yeast Two-Hybrid Skeletal Muscle Library Screening with α-Sarcoglycan.

Genbank Matched Sequences	Clone Number	Translation	Accession Number
Cold Shock Domain in dbpA	234, 342, 357, 390	In-frame	L29064-L29073
Na^+/K^+ ATPase	60	In-frame	J05096
Voltage gated sodium channel	169	In-frame	D17311
Human skeletal α-actin	177	Not in-frame	-
Tropomyosin	76	Not in-frame	-
Ribosomal protein	154	In-frame	-
Mitochondrial protein	58, 71, 75, 148	In-frame	-
Empty vector	41, 66, 159, 165, 166, 167, 168, 172, 175, 176, 178, 179, 198, 265	-	-
No match to known sequence	55, 63, 137, 156, 162, 164, 171, 173, 180	-	-

Figure 3-3. The Domain Structure of the DNA Binding Protein DbpA.

The dbpA is coded in 8 separate exons, which are indicated (E1-E8). The dbpA is divided into (i) the N-terminal region (amino acid residues 1-83), (ii) the cold shock domain (amino acid residues 83-191), (iii) the alternative domain (amino acid residues 191-260) and (iv) the C-terminal domain (amino acid residues 260-342).


E1
E2
E3
E4
E5
E6
E7
E8
1
83
191
260
342
N-terminal domain
Cold shock domain
Alternative domain
C-terminal domain

3.2.2 Evidence that α-Sarcoglycan Forms a Complex.

While testing for immunoreactivity of anti-α-SG I made an unexpected discovery. α-Sarcoglycan is a 50 kDa integral membrane protein, however when separated on a SDS-PAGE in the presence of reducing buffer after boiling or not-boiling, a difference in the mobility of the two protein samples was observed.

Figure 3-3 A, lane 1 shows purified sarcolemma proteins mixed with protein loading buffer containing β-mercaptoethanol and boiled for 10 min. A 50 kDa protein band is detected corresponding to α-sarcoglycan. However, when the sarcolemma proteins are not boiled prior to SDS-PAGE, a 100 kDa α-sarcoglycan protein band is detected (Figure 3-4 A, lane 2) and a reduction of the 50 kDa protein band is apparent.

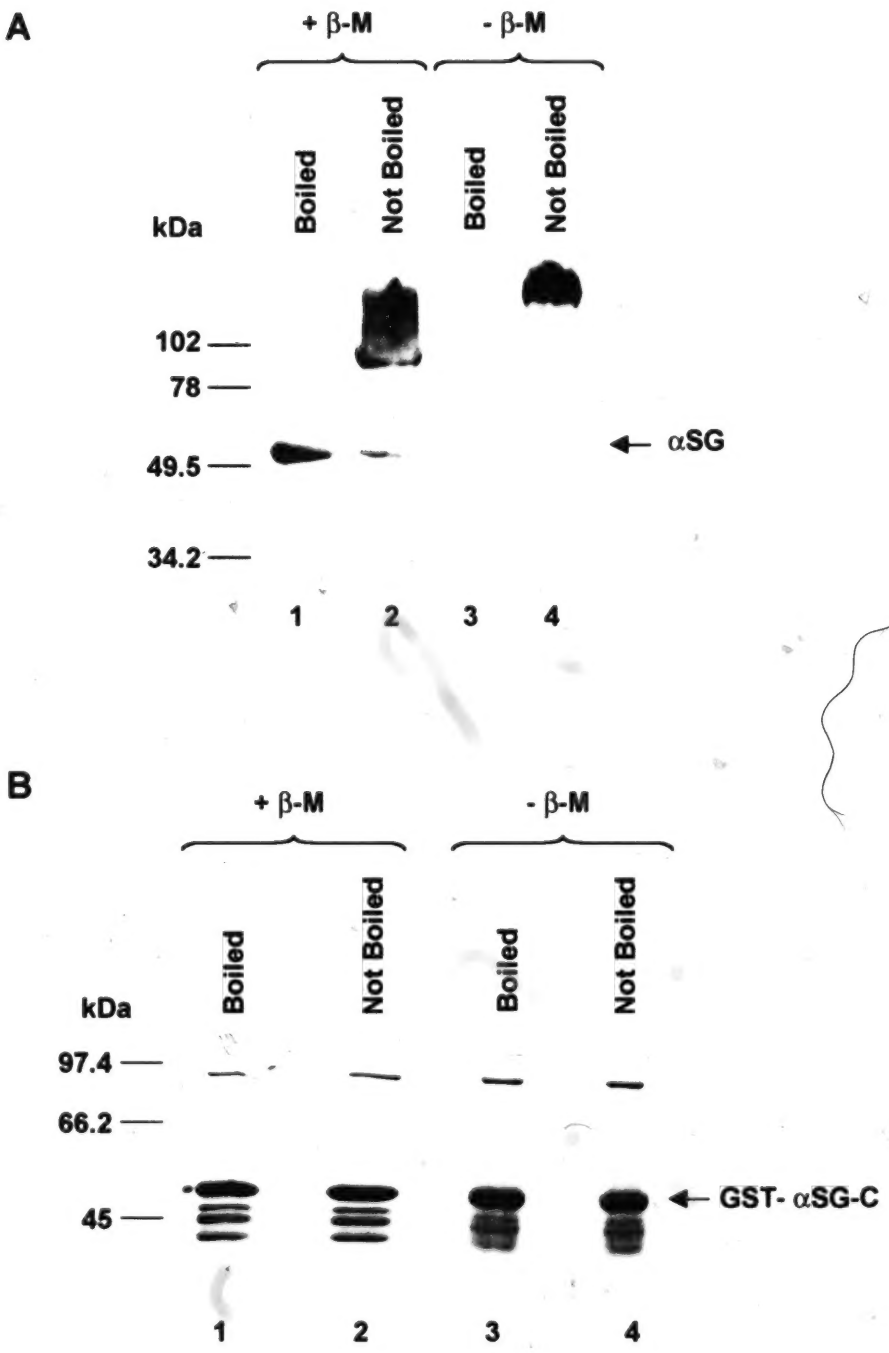
Figure 3-4 A, lane 3 shows the same sarcolemma protein preparation mixed with protein loading buffer lacking β-mercaptoethanol. When the protein mixture is boiled for 10 min both the 100 kDa and 50 kDa protein bands are detected with an anti-α-sarcoglycan antibody. Figure 3-4 A, lane 4 shows detection of only a 100 kDa protein band when the sarcolemma proteins are mixed with protein loading buffer lacking β-mercaptoethanol and not boiled.

The same assay was performed on the GST fusion protein expressing the C-terminus of α-sarcoglycan. Figure 3-4 B clearly shows that GST-αSG-C did not form a complex, which was seen for the full length protein in the sarcolemma protein preparations in Figure 3-4 A.

The 50 kDa α-sarcoglycan protein migrated at an apparent molecular weight of 100,00 when not boiled (Figure 3-4 A). There is no mobility shift when GST-αSG-C is separated on a SDS-PAGE (Figure 3-4 B), which would eliminate the C-terminus of the

Figure 3-4. Evidence for a Complex Formed by α-Sarcoglycan Protein.

A, Fourty μg of sarcolemma membrane proteins (SL) were separated by SDS-PAGE (10%). The protein samples were prepared in the presence or in the absence of β-mercaptoethanol in SDS protein loading buffer. A Western blot was performed with anti-α-sarcoglycan antibodies. Lane 1, SL proteins separated in the presence of β-mercaptoethanol and boiled for 10 min prior to loading onto the gel. Lane 2, SL vesicles separated in the presence of β-mercaptoethanol and not boiled. Lane 3, SL vesicles separated in the absence of β-mercaptoethanol and boiled for 10 min prior to loading onto the gel. Lane 4, SL vesicles separated in the absence of β-mercaptoethanol and not boiled. **B**, Fourty μg of GST-αSG-C protein was separated by SDS-PAGE (10%) and visualized by Coomassie blue staining. The protein samples were prepared in the presence or in the absence of β-mercaptoethanol in SDS protein loading buffer. Lane 1, GST-αSG-C separated in the presence of β-mercaptoethanol and boiled for 10 min prior to loading onto the gel. Lane 2, GST-αSG-C separated in the presence of β-mercaptoethanol and not boiled. Lane 3, GST-αSG-C separated in the absence of β-mercaptoethanol and boiled for 10 min prior to loading onto the gel. Lane 4, GST-αSG-C separated in the absence of β-mercaptoethanol and not boiled. The 50 kDa protein band corresponding to α-sarcoglycan and the 35 kDa protein band corresponding to GST-αSG-C are indicated by arrows. The position of the Bio-Rad molecular weight markers are indicated.


A
+ β-M
- β-M
Boiled
Not Boiled
Boiled
Not Boiled
kDa
102
78
49.5
34.2
αSG
1
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3
4
B
+ β-M
- β-M
Boiled
Not Boiled
Boiled
Not Boiled
kDa
97.4
66.2
45
GST- αSG-C
1
2
3
4

protein involved in forming a dimer. This suggests that α-sarcoglycan has the ability to form a complex, either (i) with itself, at the N-terminus or (ii) with another protein of approximately 50 kDa. The former is unlikely since Rybakova and Ervasti (1997) have shown that all the proteins present in the DGC are monomers. The bond formed in this complex is stable under β-mercaptoethanol conditions which would suggest that the bond formed is not a disulfide linkage (Figure 3-4 A, lane 2). However the bond is broken when the proteins are subjected to heat for any length of time. Since the DGC contains only one copy of each of the proteins in the complex then it is possible that a DAP is responsible for this α-sarcoglycan complex formation. One other member of the sarcoglycan family is β-sarcoglycan which is a 43 kDa protein and might bind to α-sarcoglycan. It would be interesting to obtain β-sarcoglycan antibodies to determine if this is the case.

3.3 Discussion.

Using the yeast two-hybrid assay I successfully isolated 372 positive clones encoding proteins interacting with the α-sarcoglycan C-terminal cytoplasmic tail (Figure 3-1 and 3-2). At the present time only basic characterization of these clones has been carried out. Using restriction endonuclease digestion it was possible to group a total of 72 clones as that of the Cold Shock domain in the dbpA protein, four of these clones were identified by DNA sequencing (Table 3-1).

The CSD is a highly conserved region found in Y-Box proteins (Figure 3-3). The positive clones which were analyzed for their nucleotide sequence revealed that they encoded amino acids 107-177 of dbpA, a human DNA binding protein (Table 3-1) (Kudo

et al., 1995). DbpA is a member of the Y-Box family of proteins and consists of three regions, a variable N-terminus, a highly conserved Cold Shock Domain which binds nucleic acids, and a hydrophilic C-terminal tail which contains a Casein kinase II phosphorylation site (Figure 3-3) (Kudo *et al.*, 1995). The CSD is located at exon 2-5 and codes for amino acids 106-155 of dbpA. The dbpA protein is highly expressed in skeletal muscle and the heart (Kudo *et al.*, 1995), which is the same location as that of the α-sarcoglycan protein. Further investigation will be required to establish physiological relevance of these protein-protein interactions.

A surprising result was the identification of a clone encoding a portion (amino acid residues 117-182) of a human α2 subunit of the Na^+/K^+ ATPase (Shull *et al.*, 1989) (Table 3-1). The Na^+/K^+ ATPase is an integral membrane protein. The predicted topology of the α2 subunit of the Na^+/K^+ ATPase indicates that the protein consists of ten transmembrane segments and a large cytoplasmic catalytic domain (Shull *et al.*, 1989). This catalytic domain (amino acid residues 347-785) is responsible for the hydrolysis of ATP coupled to the exchange of Na^+ and K^+ ions across the plasma membrane. This action creates the electrochemical gradient of Na^+ and K^+, providing the energy needed to transport various nutrients (Shull *et al.*, 1989).

Clone #60 encoded amino acid residues 117-182 of the α-subunit of the Na^+/K^+ ATPase. This region contains one predicted transmembrane domain (amino acid residues 128-147), preceeded by 11 amino acid residues at the extracellular side of the plasma membrane and followed by 35 amino acids in the cytoplasm (amino acid residues 148-182). It is unlikely that an integral membrane protein such as the Na^+/K^+ ATPase can be targeted to the nucleus in the yeast two-hybrid assay. However, clone #60 encodes a

short fragment of the ATPase suggesting that it could have been folded in such a way as to hide the hydrophobic residues. Even though the C-terminus of α-SG interacts with Na^+/K^+ ATPase in a system that was not designed to identify interacting membrane proteins, this could potentially be a very important finding. Future experiments with the cytoplasmic 35 amino acids (amino acid residues 148-183) of the α2 subunit of Na^+/K^+ ATPase and the C-terminus of α-SG may determine if these two proteins interact and if these interactions are physiologically relevant.

There still remains a total of 268 positive clones which have not been analyzed for their nucleotide sequence and restriction endonuclease digestion pattern.

CHAPTER FOUR

THE C-TERMINUS OF DYSTROPHIN INTERACTS WITH α-ACTININ AND ACTIN

4.1 Introduction.

It is well documented that proteins found in the DGC are drastically reduced in DMD patients when dystrophin lacks the cysteine rich region and the C-terminal domain. This C-terminal portion of dystrophin is highly conserved through evolution (Lemaire *et al.*, 1988). A study carried out by Arahata *et al.* (1991), discovered that BMD patients have dystrophin gene products which contain the C-terminal region. However, they also showed that in patients with DMD the C-terminus was absent. The clinical phenotype of DMD is much more severe than that found in BMD patients, suggesting that the C-terminal region of dystrophin is important, not only for the stability of the DGC, but may contain some specific functions unique to that region. The C-terminal domain of dystrophin binds calmodulin (Anderson *et al.*, 1996) and is also a substrate for dephosphorylation by calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase (Walsh *et al.*, 1995). Phosphorylation-dephosphorylation of dystrophin may play a role in regulating dystrophin-membrane (protein-protein) interactions and/or transducing signals from the extracellular matrix (Michalak and Opas, 1997).

In order to identify the functions of the C-terminus of dystrophin (CT-1) we carried out the yeast two-hybrid assay to identify proteins interacting with this region of dystrophin. Dystrophin is known to bind to two proteins at its C-terminus. CT-1 consists of amino acids 3,485-3,685, and binds to dystrobrevin at amino acids 3,501-3,541 (Sadoulet-Puccio *et al.*, 1997). The CT-1 region also binds to β1-syntrophin (Suzuki *et al.*, 1995). The syntrophin binding site at the C-terminus of dystrophin has been identified at amino acid 3,443-3,535 (Suzuki *et al.*, 1995). The CT-1 region does not encompass the full binding region of α1-syntrophin which is located at amino acids

3,444-3,494. However CT-1 does contain the β1-syntrophin binding site which is located at amino acids 3,495-3,535. I carried out the yeast two-hybrid library screening assay to identify new proteins interacting with the last 200 amino acids of dystrophin.

4.2 Results.

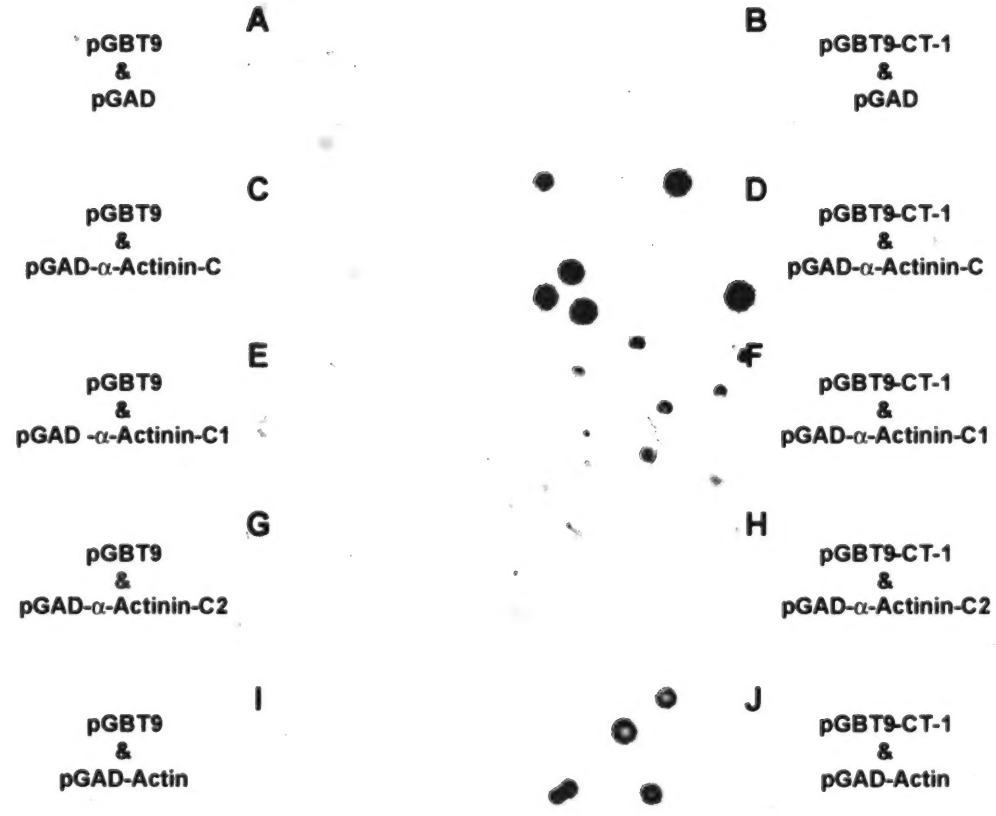
4.2.1 The C-Terminus of Dystrophin Interacts with Skeletal Muscle α-Actinin-2 and Actin in the Yeast Two Hybrid System.

The yeast two hybrid screening method was used to identify proteins interacting with the C-terminal region of dystrophin. The cDNA encoding the last 200 amino acids of dystrophin (designated CT-1) were used for screening a human skeletal muscle cDNA library. Screening the library resulted in the identification of 16 positive yeast colonies. Two of these clones (clone 5 and clone 16) were further characterized because they developed a positive blue colour within less than one hour (Figure 4-1).

Clone 5 was 1,449 nucleotides in length and nucleotide sequence analysis revealed that it corresponded to the C-terminus of human skeletal muscle α-actinin-2 (nucleotides 1,436-2,885) (Beggs *et al.*, 1992). This region encodes the last 473 amino acids of the mature α-actinin-2 protein and does not contain the actin binding site (Beggs *et al.*, 1992). Figure 4-1 D shows that co-transformation of yeast with pGAD-α-actinin-C and pGBT9-CT-1 resulted in rapid (<30 min) activation of the LacZ reporter gene. Transformation of yeast with pGAD-α-Actinin-C, pGBT9-CT-1 or control pGAD and pGBT9 vectors did not produce any colour development for at least 48 hours (Figure 4-1).

Figure 4-1. The C-Terminal Region of Dystrophin Interacts with α-Actinin-2 and α-Actin in the Yeast Two-Hybrid System.

The human skeletal muscle cDNA library was screened with the cDNA encoding the C-terminus of dystrophin. The β-galactosidase filter lift assay was performed to detect positive colonies. Identification of positive clones was determined by nucleotide sequence analysis. **A**, yeast cells co-transformed with pGBT9 and pGAD control vectors; **B**, yeast co-transformed with pGBT9-CT-1 (the last 200 amino acids of human skeletal muscle dystrophin) and pGAD; **C**, yeast co-transformed with pGBT9 and pGAD-α-Actinin-C (the C-terminal 473 amino acids of α-actinin-2); **D**, yeast co-transformed with pGBT9-CT-1 and pGAD-α-Actinin-C; **E**, yeast co-transformed with pGBT9 and pGAD-α-Actinin-C1 (amino acid residues 422-506 of human α-actinin-2); **F**, yeast co-transformed with pGBT9-CT-1 and pGAD-α-Actinin-C1; **G**, yeast co-transformed with pGBT9 and pGAD-α-Actinin-C2 (amino acid residues 507-894 of human α-actinin-2); **H**, yeast co-transformed with pGBT9-CT-1 and pGAD-α-Actinin-C2; **I**, yeast co-transformed with pGBT9 and pGAD-Actin (human α-actin); **J**, yeast co-transformed with pGBT9-CT-1 and pGAD-Actin.


A
pGBT9
&
pGAD
B
pGBT9-CT-1
&
pGAD
C
pGBT9
&
pGAD-α-Actinin-C
D
pGBT9-CT-1
&
pGAD-α-Actinin-C
E
pGBT9
&
pGAD -α-Actinin-C1
F
pGBT9-CT-1
&
pGAD-α-Actinin-C1
G
pGBT9
&
pGAD-α-Actinin-C2
H
pGBT9-CT-1
&
pGAD-α-Actinin-C2
I
pGBT9
&
pGAD-Actin
J
pGBT9-CT-1
&
pGAD-Actin

Analysis of clone 16 revealed that it was identical to human skeletal muscle α-actin, nucleotides 936-1,251 (Gunning *et al.*, 1983). Again, activation of the LacZ reporter gene was seen when pGAD-Actin and pGBT9-CT-1 vectors were co-transformed into yeast (Figure 4-1 J). Development of a strong blue colour for these interacting molecules required approximately 2 hr incubation. With the use of the yeast two hybrid system two new proteins have been identified which interact with the C-terminal region of dystrophin.

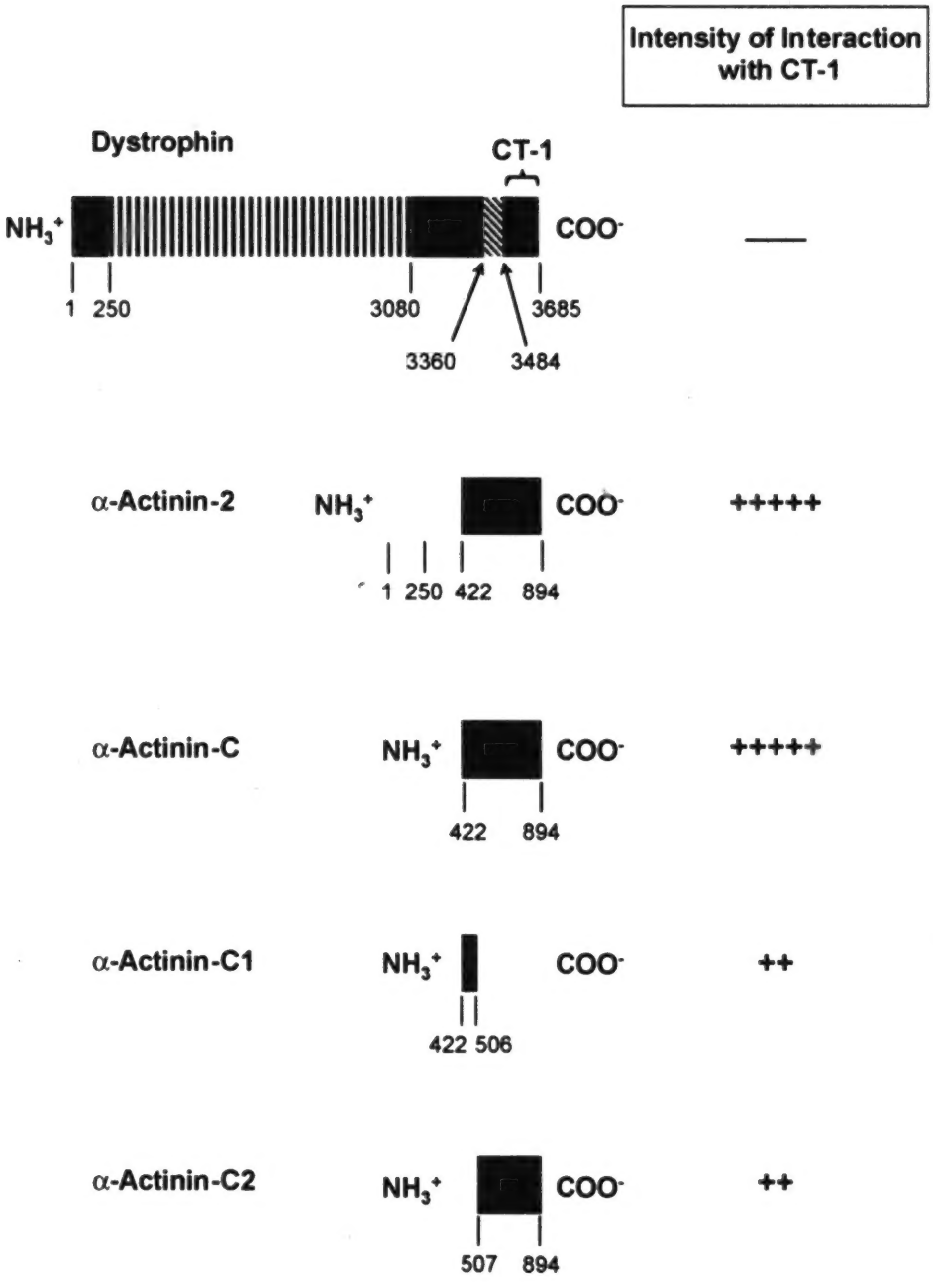
4.2.2 Interaction of α-Actinin-2 Fragments with the C-Terminus of Dystrophin.

To attempt to identify the region that α-actinin-2 is involved in binding to the C-terminus of dystrophin, the last 473 amino acids of α-actinin-2 were divided into two regions. α-Actinin-C1 (nucleotides 1,436-1,691) which is 85 amino acids in length and α-actinin-C2 (nucleotides 1,692-2,855) which is 388 amino acids in length (Figure 4-2). Both constructs pGAD-α-Actinin-C1 and pGAD-α-Actinin-C2 (Figure 2-5) were transformed into the yeast strain HF7c along with pGAD-CT-1. Transformations with pGBT9-CT-1, pGAD-α-Actinin-C1 or pGAD-α-Actinin-C2 alone did not produce any colour development during the β-galactosidase lift assay. Co-transformation with pGBT9-CT-1 and pGAD-α-Actinin-C1 or pGAD-α-Actinin-C2 both had the ability to activate the LacZ reporter gene (Figure 4-1 F and H). These reactions were compared with the complete C-terminus of α-actinin. In 3 independent experiments pGBT9-CT-1 interacted with each of the α-actinin regions C1 and C2 in a different manner.

In yeast co-transformed with pGBT9-CT-1 and pGAD-α-Actinin-C a blue colour developed within 30 minutes. When yeast were co-transformed with pGBT9-CT-1 and

Figure 4-2. Diagrammatic Representation of the Protein Fragments of Dystrophin and α-Actinin used in the Yeast Two-Hybrid System.

Dystrophin is shown with the N-terminal (amino acid residues 1-250) region, rod domain, cysteine rich region (amino acid residues 3,080-3,360) and C-terminus (amino acid residues 3,360-3,685). The portion of the C-terminus of dystrophin, designated CT-1 (amino acid residues 3,484-3,685), was used for screening the yeast two-hybrid library. α-Actinin-2 is shown in green and is divided into the N-terminal region (amino acid residues 1-250) which contains an actin binding site, the central domain, and the C-terminus (amino acid residues 422-894) which interacts with the CT-1 region of dystrophin. The C-terminus of α-actinin is shown divided into two fragments, α-Actinin-C1 (amino acid residues 422-506) and α-Actinin-C2 (amino acid residues 507-894) which were used for further analysis of protein-protein interactions. The intensity of the interaction of the CT-1 region of dystrophin with the different regions of α-actinin-2 is depicted by (+), as determined by a β-galactosidase filter lift assay.


Intensity of Interaction with CT-1
Dystrophin
CT-1
NH3+
COO-
1
250
3080
3360
3484
3685
—
α-Actinin-2
NH3+
COO-
1
250
422
894
+++++
α-Actinin-C
NH3+
COO-
422
894
+++++
α-Actinin-C1
NH3+
COO-
422
506
++
α-Actinin-C2
NH3+
COO-
507
894
++

either pGAD-α-Actinin-C1 or pGAD-α-Actinin-C2 the time for the yeast colonies to develop a blue colour was approximately 2.5 hr, which is significantly slower than the C-terminus of α-actinin (Figure 4-1). This suggests that the C-terminus of dystrophin binding to the C-terminus of α-actinin requires both the C1 and C2 segments in order for a strong interaction to occur.

In order to further quantify the protein interaction between pGBT9-CT-1 and either pGAD-α-Actinin-C, pGAD-α-Actinin-C1 or pGAD-α-Actinin-C2 a liquid culture β-galactosidase assay was used. However, only those yeast co-transformed with pGBT9-CT-1 and pGAD-α-Actinin-C utilized the substrate. The yeast which were co-transformed with pGBT9-CT-1 and either pGAD-α-Actinin-C1 or pGAD-α-Actinin-C2 were left for a total of 24 hours (the recommended time stated by the manufacturer) with no visible sign of substrate utilization. The reason for this reaction not occurring could be, simply that ONPG is not sensitive enough of a reagent to detect the interaction shown with the β-galactosidase lift assay and confirms that C1 and C2 interactions with CT-1 were weaker than the full length C-terminus with CT-1.

4.2.3 The Interaction the C-Terminus of Dystrophin with α-Actinin.

4.2.3.1 *In Vitro* Interaction Between α-Actinin-2, α-Actin and Dystrophin.

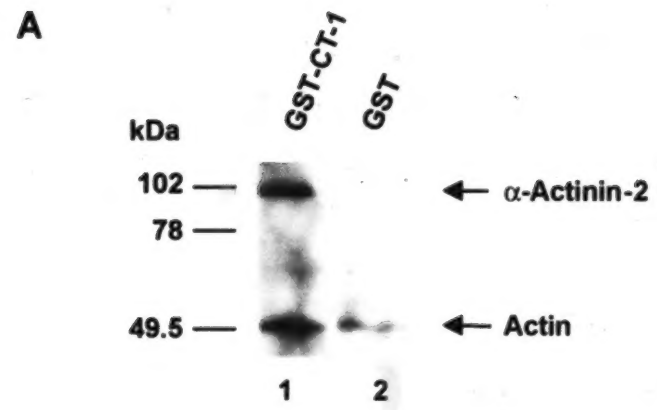
For protein-protein interaction α-actinin was purified from rabbit skeletal muscle as detailed under "Methods". GST-CT-1 was expressed and purified (Figure 2-14) as described under "Methods".

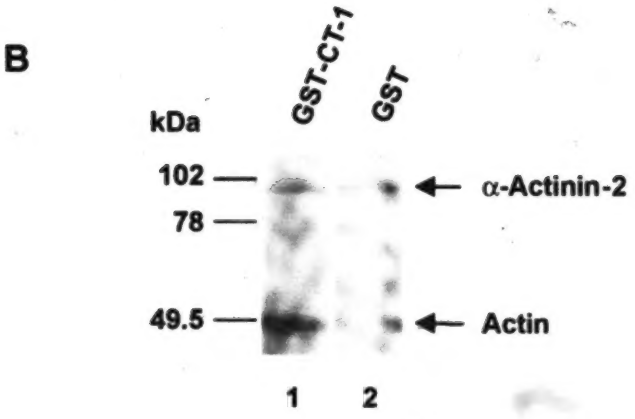
A ligand binding (blotting) method was performed to further confirm the interaction between dystrophin, actin and α-actinin-2. This method has been used in the

past to identify protein-protein interactions including identification of the low density lipoprotein receptor (Daniel *et al.*, 1983), calsequestrin and calreticulin binding proteins (Mitchell *et al.*, 1988; Burns and Michalak, 1993). Purified rabbit skeletal muscle α-actinin-2 was separated on a SDS-PAGE, transferred to nitrocellulose and incubated with purified GST-CT-1 fusion protein (Milner *et al.*, 1992). GST was used as a control. As described previously α-actinin used in this study contained bound actin. This would allow investigation of dystrophin-α-actinin-2 and dystrophin-actin interactions using the same ligand blotting experiment. Anti-dystrophin antibodies, which recognize GST-CT-1, were used to detect protein complexes containing the CT-1 region of dystrophin. Figure 4-3 shows that under these conditions GST-CT-1 interacted with both α-actinin-2 and actin. Densitometric scans revealed an increase of α-actinin binding to GST-CT-1 of 10.8 +/- 2.02 (n=3) fold, compared to GST protein alone. Interestingly, GST-CT-1 bound to α-actinin and actin in a Ca^{2+}-dependent manner. Figure 4-3 A shows this interaction was observed in the presence of 5 μM free Ca^{2+} concentrations. Figure 4-3 B shows approximately the same amount of GST-CT-1 binding to α-actinin compared to the control.

Figure 4-3. The C-Terminus of Dystrophin Interacts with α-Actinin and Actin *In Vitro*.

α-Actinin was purified from rabbit skeletal muscle as described in "Methods". This protein preparation also contains actin. The proteins were separated on a 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with purified GST-CT-1 fusion protein or with GST alone. The blots were then probed with an anti-dystrophin antibody. **A**, ligand blotting assay was carried out in the presence of 0.1 mM calcium (5 μM free calcium). Lane 1, ligand blotting with purified GST-CT-1; lane 2, ligand blotting with recombinant GST. **B**, ligand blotting assay was carried out in the absence of calcium and the presence of 0.1 mM EGTA. Lane 1, ligand blotting with purified GST-CT-1; lane 2, ligand blotting with recombinant GST. The positions of α-actinin-2, actin and the Bio-Rad molecular weight markers are indicated.


A
GST-CT-1
GST
kDa
102
78
49.5
α-Actinin-2
Actin
1
2


B
GST-CT-1
GST
kDa
102
78
49.5
α-Actinin-2
Actin
1
2

4.2.3.2 Localization of α-Actinin-2 to the Purified Plasma Membrane of Tissue Culture Cells and Purified Sarcolemma Vesicles.

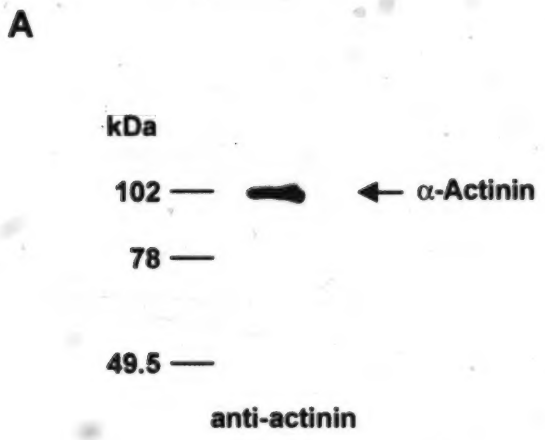
Dystrophin is a component of the sarcolemma, which is the plasma membrane of muscle cells (Minetti *et al.*, 1992; Straub *et al.*, 1992). If α-actinin and dystrophin interact in vivo these proteins should both be present in the isolated plasma membranes from C_2C_{12} cells which are a skeletal muscle myoblast cell line.

In order to test for this, sarcolemma vesicles containing dystrophin (see "Methods") were purified from rabbit skeletal muscle and tested for the presence of α-actinin-2 and dystrophin using specific anti-α-actinin-2 and anti-dystrophin antibodies. Figure 4-4 A shows a 100 kDa α-actinin-2 protein band present in the purified membrane preparation. Figure 4-4 B shows the presence of dystrophin in this sarcolemma preparation.

High purity plasma membrane preparations from C_2C_{12} muscle myoblasts in culture were also tested for the presence of α-actinin-2. It is very well established that dystrophin localizes to the plasma membrane in these cells (Belkin and Burridge, 1995). As with skeletal muscle sarcolemma the presence of α-actinin-2 was determined in these plasma membrane preparations using specific anti-α-actinin-2 antibodies. Figure 4-5 shows highly purified C_2C_{12} myoblast plasma membranes contained a 100 kDa α-actinin-2 protein band. This data shows that dystrophin and α-actinin-2 are both components of the purified skeletal muscle of C_2C_{12} muscle myoblast plasma membranes.

Figure 4-4. Identification of α-Actinin and Dystrophin in Muscle Sarcolemma.

Rabbit skeletal muscle sarcolemma vesicles were isolated as described in "Methods". Membrane proteins were separated on a 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with an anti-actinin or anti-dystrophin antibody. **A**, Western blot with anti-α-actinin-2 antibodies. **B**, Western blot with anti-dystrophin antibody. α-Actinin and dystrophin are indicated by the arrows. The position of the Bio-Rad molecular weight markers are indicated.


A
kDa
102
78
49.5
α-Actinin
anti-actinin

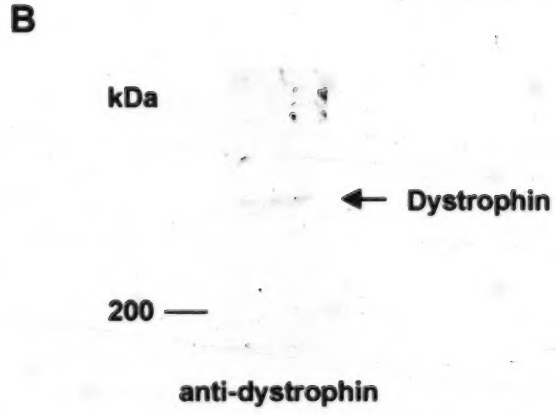
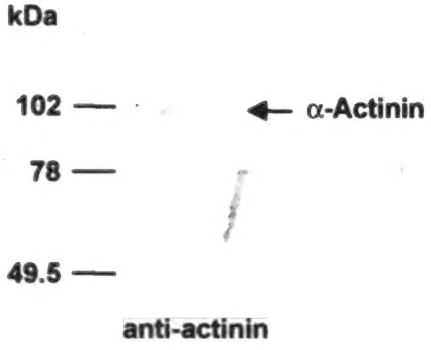

B
kDa
Dystrophin
200
anti-dystrophin

Figure 4-5. Identification of α-Actinin in C_2C_{12} Myotube Plasma Membrane Vesicles.

Plasma membrane vesicles were isolated from C_2C_{12} myoblasts by a colloidal silica beads method as outlined under "Methods". Membrane proteins were separated on a 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-α-actinin-2 antibodies. The position of α-actinin-2 and the Bio-Rad molecular weight markers are indicated.


kDa
102
78
49.5
α-Actinin
anti-actinin

4.2.3.3 Immuno Co-Localization of Dystrophin and α-Actinin in Skeletal Muscle Fibres.

Double label immunofluorescence confocal microscopy was employed to determine whether dystrophin and α-actinin-2 co-localized at the microscopic level. Sections through the plane of the plasma membrane revealed a patchy pattern of dystrophin staining where gaps represented areas above or below the plane focus (Figure 4-6 A). Dystrophin staining exhibits a periodicity reminiscent of the pattern of Z lines revealed by staining of α-actinin-2 (Figure 4-6 B). Superposition of the two images demonstrates that these represent areas of co-localization of dystrophin and α-actinin-2 where the Z lines meet at the plasma membrane (Figure 4-6 C). A second periodic focus of dystrophin staining appears to alternate with the Z lines at the location of the M lines.

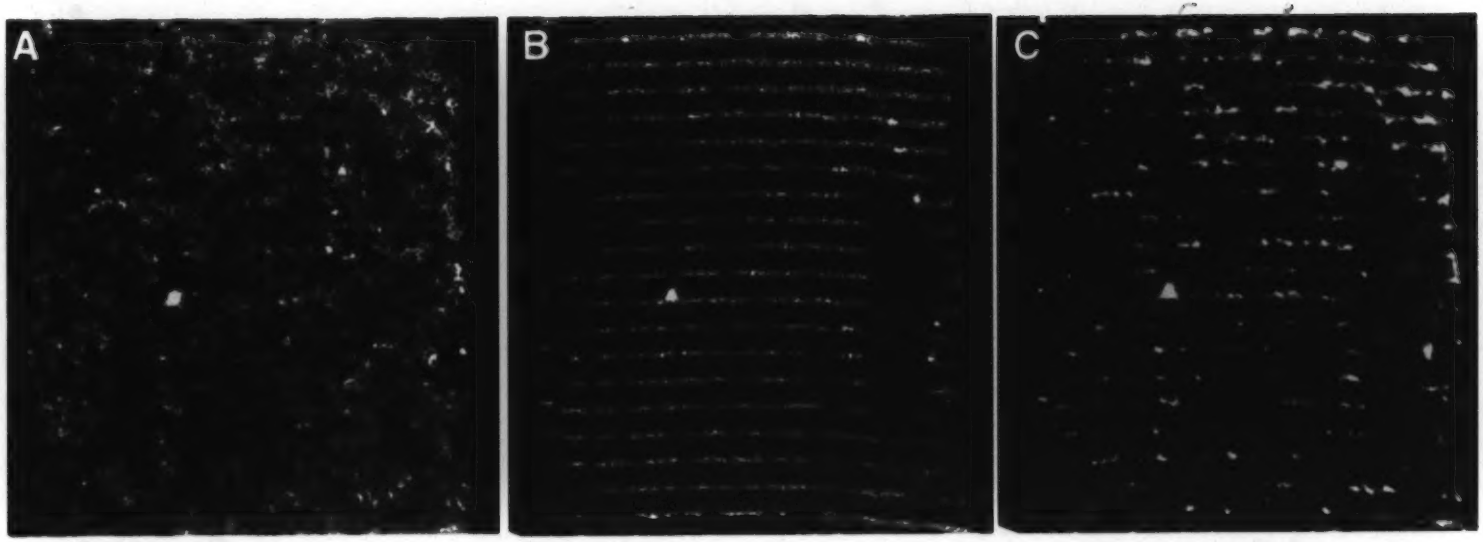
4.3 Discussion.

Using the yeast two-hybrid method we identified α-actinin-2 and α-actin as dystrophin binding proteins (Figure 4-1). The α-actinin protein was divided into two fragments, C1 and C2 which were also shown to bind to CT-1 but with lower affinity to that of full length α-actinin. This was determined by the slower β-galactosidase reaction time when a lift assay was performed with the C1 and C2 fragments. This indicates that dystrophin needs both fragments in order to bind to α-actinin, or that the binding site is located at the end of C1 and the beginning of the C2 fragments.

The results obtained from the yeast two-hybrid assay were confirmed by a ligand blotting technique, by co-localization of dystrophin and α-actinin-2 in isolated skeletal

Figure 4-6. Co-localization of Dystrophin and α-Actinin-2 in Skeletal Muscle.

Confocal immunofluorescence micrographs of human trapesius muscle stained with anti-dystrophin (A) and anti-α-actinin-2 (B) (both pseudocoloured) and combined images (C) demonstrating co-localization (yellow) where red α-actinin and green dystrophin signals overlap. Elongated oval structure at right is a nucleus.


A
B
C

muscle sarcolemma vesicles and in the plasma membranes isolated from C_2C_{12} myoblasts, and also by indirect immunolocalization of dystrophin and α-actinin-2 in skeletal muscle cells.

There are two important consequences to these findings: (i) actin molecules may have multiple attachment sites on dystrophin both direct and indirect (via α-actinin-2) affecting the geometry and function of the DGC, and (ii) α-actinin-2 binding to the C-terminus of dystrophin provides a new and important site of attachment for the DGC to other cytoskeletal proteins and a connection to adhesion complexes made up of integrin, vinculin, talin and tensin.

It is well established that dystrophin is a cytoskeletal protein that binds actin and interacts with a DGC to bridge the cytoskeleton, the basal lamina and the plasma membrane. My findings add new components to the DGC. In Figure 5-1 I propose a new model for the DGC organization that includes α-actinin-2 and actin interactions with dystrophin reported here. Dystrophin is organized into distinct structural domains with the N-terminal domain binding F-actin (Anderson *et al.*, 1996). New F-actin binding sites have recently been discovered in the middle of the rod domain of dystrophin (Rybakova *et al.*, 1996; Rybakova and Ervasti, 1997). It has also been suggested that F-actin runs along side the dystrophin molecule in a parallel fashion. With the data showing actin binding to the C-terminus of dystrophin it is possible to postulate that the F-actin molecule runs the length of dystrophin, binding at the N-terminus, rod domain and the C-terminus. Here I show that the C-terminal domain of dystrophin binds to α-actinin-2 and actin and, therefore, provides additional site(s) for F-actin attachment to the protein. I propose that dystrophin forms lateral, multi-contact association with actin.

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

The major findings of my research are: (1) the identification of candidate muscle proteins that interact with the C-terminal, cytoplasmic domain of α-SG; (2) the discovery that α-actinin-2 is a new component of the DGC and (3) that actin binds to multiple sites on dystrophin. Figure 5-1 shows a revised model of the DGC including findings of my research. By comparing the DGC model before I undertook this research (Figure 1-2) to the newly revised model encompassing my findings (Figure 5-1), there are a number of novel protein-protein interactions with members of the DGC. These results have suggested the re-designing of the DGC and sparked many new ideas as to the function of members of the DGC in the muscular dystrophies. I have shown the C-terminus of α-sarcoglycan and the C-terminus of dystrophin are important regions for (i) protein-protein interactions, (ii) roles in possible signaling pathways, (iii) maintaining the integrity of the sarcolemma membrane and (iv) possible areas for gene therapy.

5.1 α-Sarcoglycan Protein Interactions.

One important observation made was the C-terminus of α-SG interacting with the cytoplasmic protein dbpA. DbpA is a member of the Y-box family of proteins. The eukaryotic Y-box proteins were characterized because of their interaction with DNA containing the CAAT sequence (Ozer *et al.*, 1990; Gai *et al.*, 1992). This regulatory sequence is found in the transcriptional control regions of a variety of eukaryotic genes, including the major histocompatibility complex class II genes (Dorn *et al.*, 1987). The binding of Y-box proteins can result in either activation or repression of transcription (Wolffe, 1994; Ladomery and Sommerville, 1995). Many Y-box proteins have roles in cell proliferation, and bind to the CCAAT sequences of several growth associated genes

such as thymidine kinase (Lipson *et al.*, 1989; Gai *et al.*, 1992), proliferating cell nuclear antigen (Travali *et al.*, 1989), DNA polymerase α and Epidermal growth factor receptor (Sakura *et al.*, 1988; Pearson *et al.*, 1991). A region known as the cold shock domain is found in all Y-box proteins, this region shows approximately 90% amino acid identity between the cold shock domains found in all Y-box proteins (Graumann and Marahiel, 1995). The cold shock domain in prokaryotic Y-box proteins was so named because it regulates a cold shock stress response when cells are subjected to a reduction in temperature, initiating a reduced translation and transcription level (Goldstein *et al.*, 1990; Wolffe, 1994).

The dbpA protein is a member of the Y-box family of proteins and is expressed specifically in cardiac and skeletal muscle, a tissue distribution characteristic for α-SG (Kudo *et al.*, 1995). α-SG interacts with the CSD in the dbpA, a region which is highly conserved through evolution from procaryotes to eukaroytes. The main function of the dbpA is regulation of transcription (Ohmori *et al*, 1995; Bayarsaihan *et al.*, 1996; Ohmori *et al.*, 1996; Swamynathan *et al.*, 1997; Didier *et al.*, 1998). DbpA has been localized to both the cytoplasm and the nucleus (Kudo *et al.*, 1995) suggesting that dbpA may shuttle between the plasma membrane and the nucleus. It is tempting to speculate that α-SG may act as a communication link between the extracellular matrix and dbpA. α-SG interaction with the dbpA may lead to activation of dbpA dependent transcription pathways. When this signal is lost, for example in the case of patients suffering from SCARMD, the transcriptional signal is no longer functioning and transcription of the dbpA-dependent genes may be impaired leading to the pathology of SCARMD. This

may be invaluable for our understanding of the molecular pathology of SCARMD and for the development of potential therapies.

The C-terminus of α-SG also interacts with a short region of the Na^+/K^+ ATPase in the yeast two-hybrid system. This region of the Na^+/K^+ ATPase corresponds to the second transmembrane segment, a short extracellular region and a long cytoplasmic tail, likely interacting with α-SG. If α-SG indeed interacts with the Na^+/K^+ ATPase in cardiac cells, this interaction may effect the Na^+ and K^+ balance across the sarcolemma membrane. Cardiomyopathy is a common feature of SCARMD patients and it is not known why the loss of expression of α-SG in cardiac tissue results in cardiomyopathy. The loss of α-SG may trigger an imbalance of the regulated Na^+ and K^+ fluxes in the cardiac cells and result in cardiomyopathy.

The interaction of the Na^+/K^+ ATPase and the dbpA with the C-terminus of α-SG are both fascinating discoveries. These protein-protein interactions open up new hypothesis to important functions that α-SG may play in the sarcolemma. To date there have been no reported functions for α-SG. It is known that there is a loss of β-, γ-, and δ-sarcoglycans in the sarcolemma when α-SG is not expressed, resulting in a loss of membrane stability. My results suggest that α-SG may also be a membrane receptor and play a role in signal transduction. Further investigations need to be carried out to determine if these protein-protein interactions occur *in-vitro*.

5.2 Multiple Actin Sites on Dystrophin.

A direct binding site on dystrophin was discovered for actin in the yeast two-hybrid system. This discovery alters the DGC complex and I propose that actin runs

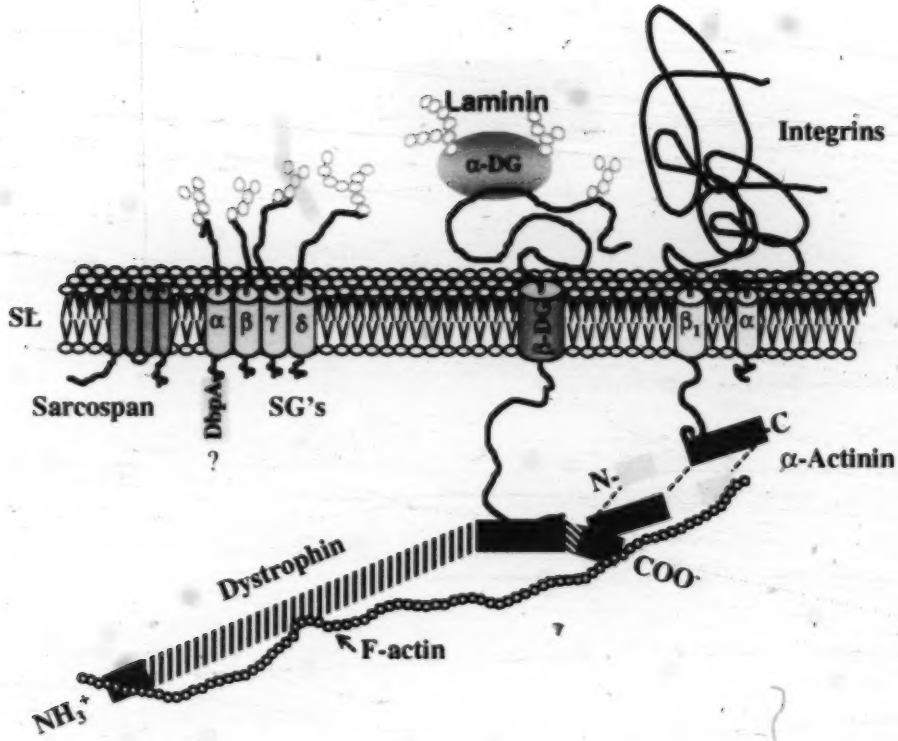
along side dystrophin with binding sites at the N-terminus, the rod domain (Rybakova *et al.*, 1996) and the newly discovered C-terminus binding site. The interaction of dystrophin with actin in this manner may provide additional anchorage sites for other cytoskeletal proteins and the extracellular matrix, thereby re-inforcing dystrophins role in maintaining the integrity of the muscle myofibril.

5.3 α-Actinin-2, a New Component of the DGC.

A major finding in this research was the discovery that the C-terminus of α-actinin bound to the C-terminus of dystrophin both *in-vitro* and *in-vivo* by the yeast two-hybrid assay, ligand blotting technique, co-localization of dystrophin and α-actinin-2 in the isolated skeletal muscle sarcolemmal vesicles and in the plasma membranes isolated from C_2C_{12} myoblasts, and by indirect immunolocalization of dystrophin and α-actinin-2 in skeletal muscle cells. α-Actinin is an actin binding protein and is a major component of the Z discs in cardiac and skeletal muscle. α-Actinin may function as an actin bundling protein or as a linking protein attaching actin filaments to various intracellular proteins. α-Actinin is divided into three domains, an N-terminal actin binding domain, four internal 120 residue repeats, and a C-terminal region containing two EF hand calcium binding motifs (Blanchard *et al.*, 1989). There are at least four α-actinin genes in humans, two skeletal isoforms (α-actinin-2 and -3) and two non-muscle isoforms (α-actinin-1 and -4) (Honda *et al.*, 1998). α-Actinin-2 is found in both skeletal and cardiac muscle, unlike α-actinin-3 which is only expressed in limb skeletal muscle. Native α-actinin forms anti-parallel homodimers (Gilmore *et al.*, 1994) or heterodimers (Chan *et al.*, 1998). In skeletal muscle α-actinin-2 can form a dimer with α-actinin-3 or with itself

Figure 5-1. A Proposed Model of the Dystrophin Glycoprotein Complex.

Model of the dystrophin glycoprotein complex showing new sites of attachment for actin and α-actinin to the C-terminus of dystrophin. α-Actinin-2 may exist as a homodimer or heterodimer (Chan *et al*., 1998) with sites interacting with dystrophin, actin and integrin complexes. The DGC is also composed of dystrophin, dystroglycans (α- and β-DG); sarcoglycans (α-SG; β-SG; γ-SG; δ-SG) and sarcospan. F-actin interacts with the N-terminus of dystrophin and with the central rod domain of the protein (Rybakov *et al*., 1996), but my work shows that it also interacts with the C-terminal domain either directly or indirectly via α-actinin.


Extracellular Matrix
Laminin
α-DG
Integrins
SL
α
β
γ
δ
β-DG
β1
α
Sarcospan
DtnpA
SG's
?
C
α-Actinin
N
Dystrophin
COO
F-actin
NH3+

as shown in Figure 5-1. α-Actinin is a component of the focal adhesion complex and directly binds to the integrins (Otey *et al.*, 1990). α-Actinin interacting with the C-terminus of dystrophin is an interesting discovery since α-actinin is an extremely important protein in the cytoskeleton and is known to interact with the integrin subunit which is affected in muscular dystrophy patients.

The exciting discovery identifying α-actinin-2 as a member of the DGC leads to a better understanding of the role dystrophin plays as a cytoskeletal protein and identification of a potential link to the integrins via α-actinin (Figure 5-1) (Otey *et al.*, 1990). In addition, α-actinin interacting with dystrophin provides an additional site for actin binding at the C-terminus of dystrophin, since α-actinin binds to actin at its N-terminus.

5.4 What are the Functional Consequences of the Interaction Between Dystrophin and α-Actinin?

Figure 5-1 shows that α-actinin may provide a link between the DGC and the integrin cytoskeleton complex. DMD patients and *mdx* mice exhibit increased expression of α7β1 (Hodges *et al.*, 1997). In skeletal muscle the α7β1 integrin is the primary laminin receptor linking the extracellular matrix with the cell cytoskeleton. One major role of integrin is to maintain the muscle integrity by linking laminin with the cytoskeleton via vinculin, talin, and α-actinin. α-Actinin may play a role as a connecting molecule between the DGC and the integrins. When the communication link between the DGC and the integrins is disrupted, the expression of the integrins may be up-regulated to

overcome the dystrophin deficiency. The up-regulated integrins may help maintain muscle function and prevent muscle cell necrosis.

5.5 Congenital Muscular Dystrophy Patients show Decreases in the Expression of α7β1 Integrins and α-Actinin-3.

A common connection between all forms of muscular dystrophies is the mutation or absence of proteins in the DGC which play an important role in maintaining the integrity of the sarcolemma and ultimately the muscle myofibril. Mutations in the α2 laminin chain gene results in Congenital muscular dystrophy (CMD) and a marked reduction in the expression levels of α7β1 integrins (Sunada *et al.*, 1994; Xu *et al.*, 1994). The α7 deficient mouse develops a similar phenotype (Mayer *et al.*, 1997). The β integrin subunit binds to laminin and seems to have a similar function as the dystrophin/DAP complex in maintaining the muscle sarcolemma. The dystrophin/DAP complex anchors the actin cytoskeleton to the basal lamina via dystrophin. The integrins interact with laminin extracellularly and actin, via α-actinin intracellularly, thereby maintaining sarcolemma stability. Both dystrophin and the integrins are found concentrated at myotendinous junctions in muscle cells and they both form links between the actin cytoskeleton and the extracellular matrix. This indicates a potential connection or relationship between the DGC /dystrophin and the integrins.

A subset of patients with CMD were found to be deficient in α-actinin-3 (North and Beggs, 1996). It was not determined if these patients had a mutation in a gene encoding α-actinin-3 or if the observations were a result of a secondary effect of the loss of α2 laminin chain expression (North and Beggs, 1996). Surprisingly, these patients

showed normal expression of α-actinin-2 (North and Beggs, 1996). This study suggests that the interaction between α-actinin-2 and the β1 integrin subunit is not affected by the loss of the α2 laminin subunit. But CMD patients have been shown to have reduced integrin expression which would suggest that α-actinin-2 expression levels would also decrease, but this does not seem to be the case (North and Beggs, 1996). There may be a sufficient number of α-actinin binding sites with other cytoskeletal proteins in the sarcolemma to maintain the stable expression of this protein.

5.6 Is there a Bi-Directional Communication Link between the Integrins and the Dystrophin/DAP Complex?

Immunofluoresence studies have shown the co-localization of dystroglycan, dystrophin, integrin β1 subunit, vinculin, talin and spectrin at costameres (Pardo *et al.*, 1983; Terracio *et al.*, 1989; Porter *et al.*, 1992). Anti-dystrophin or anti-α-sarcoglycan co-precipitated with the integrins, along with other focal adhesion proteins such as vinculin, talin, paxillin, and focal adhesion kinase (Yoshida *et al.*, 1998). α-Actinin binds specifically and with high affinity, *in-vitro*, to the β1 peptide of integrin (Otey *et al.*, 1990). The integrin cytoplasmic domain binds to the coiled coil conformation found in α-actinin. Interaction of integrin with α-actinin, links dystrophin and the DAPs to the integrins. The loss of expression of α-sarcoglycan in the sarcoglycan complex is enough to greatly reduce the adhesive properties of the focal adhesion proteins (Yoshida *et al.*, 1998). As shown in Figure 5-1 the C-terminus of α-actinin binds to the C-terminus of dystrophin, providing a communication link between the integrins, the DGC and other cytoskeletal molecules at the sarcolemma.

The results from my research provide additional evidence suggesting a communication link between dystrophin/DAP and the integrins via α-actinin. Further research on the relationship between the DGC and the integrins needs be be performed in order to understand the specific functions of these protein-protein interactions, and ultimately the correct diagnosis, early detection and gene therapies for these muscular dystrophies.

5.7 Final Conclusions.

(1) This study has identified the C-terminus of α-sarcoglycan binds to the Cold shock domain of dbpA in the yeast two-hybrid system, suggesting that α-sarcoglycan may play a role in signal transduction.

(2) Human α-actin interacts with the C-terminus of dystrophin in the yeast two-hybrid system and in protein overlay assays.

(3) The C-terminus of dystrophin interacts with α-actinin-2 both *in-vivo* and *in-vitro*.

(4) The results in this study indicate an additional actin binding site on dystrophin, directly or indirectly via α-actinin indicating that actin runs along side the dystrophin molecule.

(5) The interaction of dystrophin with α-actinin suggests a possible communication link with a group of adhesion proteins, the integrins.

Further characterization of the interaction of dystrophin with α-actinin and actin and the role that integrins play in this complex will contribute to a better understanding of the function of dystrophin and the DGC in muscular dystrophies.

CHAPTER SIX

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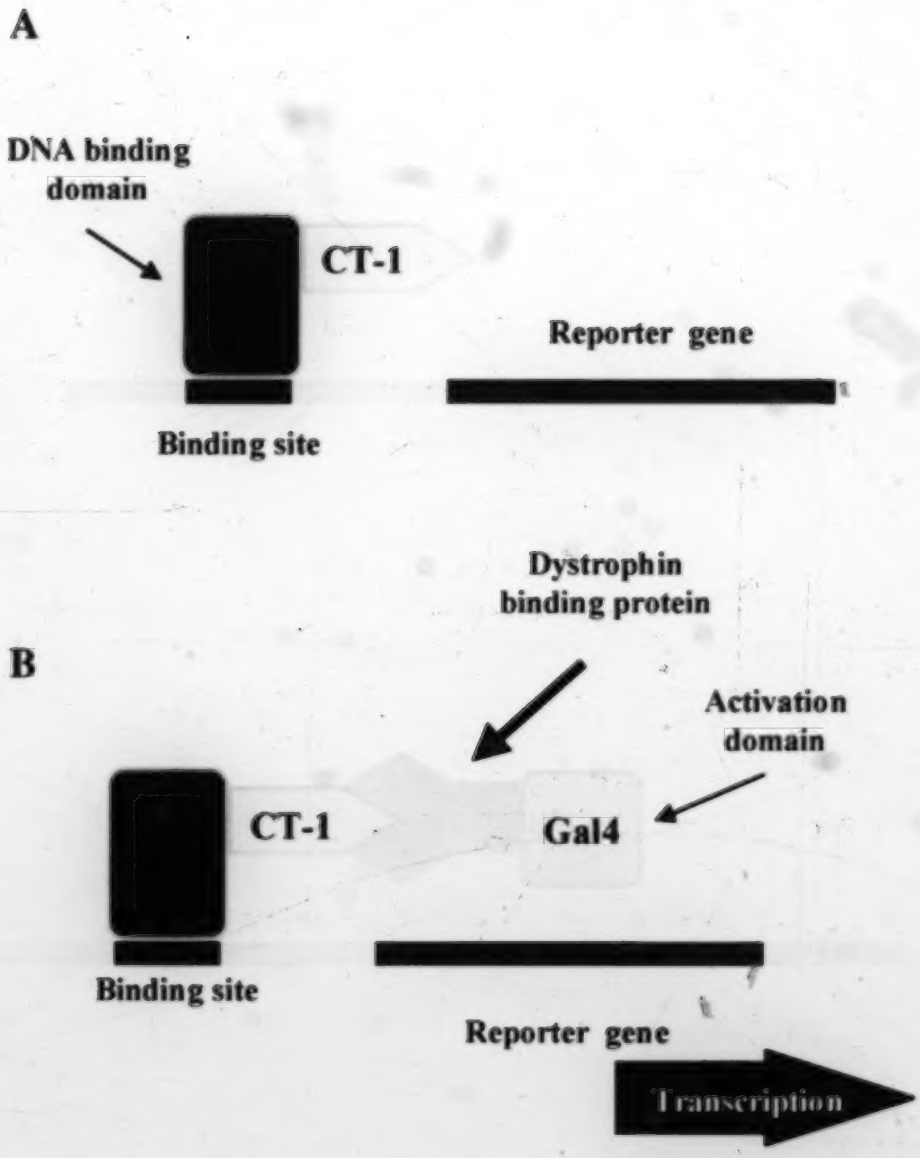
APPENDICES

Appendix 1. The Yeast Two-Hybrid Assay.

A, The CT-1 region of dystrophin was cloned in frame with the GAL4 DNA binding domain vector pGBT9. **B**, A human skeletal muscle cDNA library was cloned in frame with the GAL4 DNA activation domain vector pGAD424. If a cDNA library clone interacts with the CT-1 region of dystrophin activation of the reporter gene occurs which can be measured by a β-galactosidase filter lift assay.



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